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(54) Title: PLANT TISSUE CULTURE METHOD OF REGENERATION AND PROCEDURES FOR ISOLATING PROTOPLASTS

(57) Abstract

The invention relates to a method of regeneration of plant materials by cocultivation with microspores or anthers or cultivation in a medium containing extracts of microspores or anthers or a medium in which there has been cultured microspores or anthers or a medium containing fractions of a medium in which there has been cultured microspores or anthers. Further the invention relates to a method of isolating egg protoplasts and synergid protoplasts from ovules by mechanical dissection and a method for producing transgenic plants, homozygous plants, hybrids or cybrids and the plants, hybrids and cybrids produced by the method.

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Plant tissue culture method of regeneration and procedures for isolating protoplasts.

The present invention relates to a plant tissue culture method of regeneration of plants from protoplasts, cells or tissues, a medium to be used for regeneration of protoplasts, cells and tissues, a first method of generation of transgenic plants, a first method of generation of hybrids or cybrids, a first method of generation of homozygous plants, a first method of generating plants with special properties as well as transgenic plants, hybrids, cybrids, homozygous plants and plants with special properties as well as parts of and progeny from these transgenic plants, hybrids, cybrids, homozygous plants and plants with special properties.

The invention relates in addition to a method of isolation of egg protoplasts from ovules, a method of isolating synergid protoplasts from ovules, egg protoplasts and synergid protoplasts isolated by these methods, a second method of generating transgenic plants, a second method of generating hybrids and cybrids, a second method of generating homozygous plants, a second method of generating plants with special properties and transgenic plants, hybrids, cybrids homozygous plants and plants with special properties generated by the methods and parts of plants and progeny from the transgenic plants, hybrids, cybrids, homozygous plants and plants with special properties.

The invention further provides a method of transformation of fertilized egg cells or small embryos, a third method of generating transgenic plants and the transgenic plants generated by the method and parts of and progeny from these transgenic plants.

Today there exists for the higher plants, i.e., the gymnosperms and the angiosperms, a very large number of techniques for cultivation of protoplasts, single cells, cell aggregates and tissues as well as techniques for regeneration of plants from these structures. These techniques have allowed for the development of a series of additional techniques with basic scientific or commercial objectives such as the transfer of genetic material to plants (transformation), formation of somatic hybrids or cybrids by fusing protoplasts or protoplasts and cytoplasts (protoplasts without a nucleus) between species or genera, where sexual hybridization normally is not possible as well as selection in vitro on cells or tissues for desirable characteristics (see f. ex. Handbook of Plant Cell Culture vol. 1-4, 1. edition, 1983-1986)

The techniques developed are, however, characterized by a number of limitations. For each new plant species or variety to be cultured and regenerated, it is usually necessary at a purely empirical level to identify the optimal media and hormone concentrations. The selection of cell types that can be regenerated is based only on morphological characteristics and since the cells often are cultured for very long periods of time, a number of changes are induced at the gene, chromosome or genome level, which in the regenerants and the progeny are revealed as so called somaclonal variation. It is therefore generally necessary to perform an extensive breeding program on the regenerated plants to remove these induced defects.

It is a very essential problem that these techniques only function in an optimal way in relatively few species, in particular species belonging to the Solanaceae, and in these species only in a few genotypes. It is therefore often very difficult to implement these techniques on plants of commercial interest and in particular the cereals.

The majority of the plants, which are essential for the human consumption, belong to a group of plants known as the Gramine. Gramineae (Poaceae) is from a commercial point of view the most important family within the monocotyledonous plants. Examples of important subfamilies and genera within the Gramineae are given below:

Subfamily	Genus
Bambusoideae	Bamboo
Andropogonoideae	Saccharum
	Sorghum
	Zea
Arundineae	Phragmites
Oryzoideae	Oryza
Panicoideae	Panicum
	Pennisetum
	Setaria
Pooideae (Festuciadeae)	Poa
	Festuca
	Lolium
	Trisetum
	Agrostis
	Phleum
	Dactylis
	Alopecurus
	Avena
	Triticum
	Secale
	Hordeum

A very essential tissue culture problem for the grasses appears to be the lack of the so called wound response, i.e., that differentiated cells are unable to dedifferentiate and subsequently undergo somatic embryogenesis (Potrykus 1991). This appears to have the additional consequence that the grasses cannot be transformed by Agrobacterium (Potrykus 1991). It has for this reason been necessary for the grasses to use physical methods for gene transfer such as polyethylene glycol mediated transformation or electroporation of protoplasts or particle bombardment of intact cells (Potrykus 1991).

A number of alternative techniques for the physical introduction of DNA into plant cells and tissues has also been attempted, including microinjection of DNA into the apical meristems of immature embryos (patent application no. 990/89) or isolation of embryos from dry seeds followed by an imbibition of DNA containing solutions into the mature dry embryo (patent application WO 88/09374). Unequivocal evidence for stable transformation has, however, not been obtained by using these techniques (Potrykus 1990, 1991).

In the grasses protoplasts regeneration is only possible from protoplasts isolated from embryogenic cell suspensions (Vasil 1988, Potrykus 1990, see also patent application 1099/89 K3). In most cases calli are induced from immature zygotic embryos which subsequently are used for establishing suspensions in liquid culture. On the basis of morphological criteria, calli can be divided into a number of different types and it is possible in maize and rice to define callus types, which are optimal for establishment of suspensions cultures. In maize this particular embryogenic type of callus are formed in a frequency of 2% (Shillito et al. 1989). After the initiation of the suspension culture an elaborate selection is performed over the following months for the optimal type of cell aggregate, which must consist of relatively few cytoplasm rich cells who are in a dedifferentiated state but have maintained an embryogenic capacity. This and similar techniques are described in DK patent applications no. 1099/83, no. 2632/87, no. 6378/87, EP patent application no. 469 273 and DE Publication no 37 38 874.

Several of the techniques described above have turned out to be non-useable or only useable in rare cases

It has been shown, however, that it is possible to regenerate rice protoplasts to fertile plants, including transgenic rice from different genotypes of both the indica and the japonica varieties (Datta et al. 1990, Shimamoto et al. 1989, Zhang et al. 1988, Toryiama et al. 1989, Tada et al. 1990).

It has likewise in maize been possible to regenerate plants from protoplasts (Rhodes et al. 1988, Armstrong et al. 1989, Lyznik et al. 1989, Prioli and Söndahl 1989, Shillito et al. 1989, Morocz et al. 1990, Kamo et al. 1986).

In the grasses it has also in a few cases been possible to fuse protoplasts from closely related species and to regenerate plants from the fusion products (Oryza sativa x Echinochloa oryzicola, Terada et al. 1987; Triticum monococcum x Pennisetum americanum, Vasil et al. 1988; Festuca arundinacea x Lolium multiflorum, Takamizo et al. 1991).

Furthermore it has been possible to regenerate a few, partially fertile plants from protoplasts isolated from suspensions established from microspore derived calli of barley (Jähne et al. 1991).

It has thus been shown that the techniques described above have been used successfully on protoplasts from particular sources of material from particular species and varieties.

It is, however, not yet possible to predict from what sources of material and from which varieties protoplasts can be regenerated. It has f. ex not yet been possible to regenerate protoplasts of the egg cell to fertile plants.

Due to the difficulties encountered with regeneration from protoplasts of grasses, it has been attempted to develop techniques for the transformation of intact cells. The only method, which so far has worked, has been particle bombardment of embryogenic suspensions of maize (Gordon-Kamm et al. 1990, Fromm et al 1990) or immature zygotic embryos of rice (Christou et al. 1991). The cell aggregates or the immature zygotic embryos are bombarded in a so called particle gun with gold or tungsten particles, which have been coated by DNA. The DNA constructs contain in addition to the gene of primary interest, a selectable gene giving resistance to antibiotics such as kanamycin or hygromycin or a herbicide such as glyphosate, chlorsulfuron or Bialaphos/phosphinothricin. The genes may be placed on the same or on separate plasmids.

Selection on the bombarded aggregates is very critical since only one or a few of the cells in the aggregate are transformed. The selection therefore has to be performed using conditions allowing for the proliferation of the transformed resistant cells of the aggregate whereas the growth of the untransformed cells should be retarded and the cells eventually die. In the case of a too rapid selection, all the cells in the aggregate may die. After

bombardment of immature embryos a secondary embryogenesis is induced and a similar complex selection is performed on the secondary embryos.

Once a population of cell aggregates or secondary embryos has been established on selective medium, a regeneration of the aggregates or embryos to plants is initiated. The plants are thereafter analyzed for transformation by biochemical analyses of the enzyme conferring the resistance as well as molecular analyses of whether the introduced gene is integrated in the chromosomes of the host plant.

The regenerated plants are grown to maturity and the progeny analyzed after selfing or crossing to wild type plants to establish if the introduced gene is inherited according to the Mendelian laws for chromosomal genes. This is the final test for that transgenic plants have been generated since the biochemical and molecular analyses of the primary regenerants not always provide sufficient evidence. False positives have often been obtained where the analyses indicate that transformation has occurred while the later genetic studies show that this is not the case.

In barley a series of studies have shown that isolated embryos can first be grown into plants when the embryo is longer than 200 μm. At this stage the embryo consists of several thousand cells and a distinct apical meristem and scutellum has formed (Norstog 1961, Cameroun-Mills and Duffus 1977). It has not yet been possible by particle bombardment or microinjection to transform the few cells in the apical meristem that give rise to the tillers (Potrykus 1991).

In barley and wheat the tissue culture techniques are not as advanced as in rice and maize and it has not been possible with the same degree of precision to identify callus types from zygotic embryos or microspore derived embryos which optimal characteristics for culture, transformation and regeneration. In barley as well as in wheat it has been possible to establish suspension cultures from immature zygotic embryos or microspore derived embryos (barley: Lührs and Lörz 1987, 1988, wheat: Vasil et al. 1990a, b) and to transform these by particle bombardment or by transformation of protoplasts (Lazzeri et al. 1991, Vasil et al. 1991), but transgenic plants have not been regenerated from these. Plants can be regenerated from untransformed protoplasts of suspension cultures of these two species but with a very low frequency and the regenerants are nearly completely sterile (Vasil et al. 1990 b, Chang et al. 1991, Jähne et al. 1991, Quisheng et al. 1990). In wheat, however, it has recently been possible to regenerate transgenic plants from callus structures transformed by particle bombardment (Vasil et al. 1992). Five to seven months old embryogenic callus of the so called type C, 1-3 mm in diameter was bombarded with DNA constructs conferring resistance to phosphinothricin. Four transformed lines were obtained and plants regenerated by somatic embryogenesis on selective medium containing phosphinothricine. From two of these lines structures with roots were obtained. The cultures were at this stage 12-15 months old. From one of the other two lines were regenerated more than 100 plants of which 40 were grown to maturity. By selfing and crossing of these plants were obtained 10 kernels. Two plants were regenerated by embryo rescue from the five kernels obtained by selfing and two plants were grown from the five kernels from the cross pollinated material. Two of the four analyzed R1 plants proved to be transgenic and genetic analyses of these showed that the introduced gene was inherited as a Mendelian gene.

A number of alternative techniques has also been used for transformation and regeneration of egg cells. DNA was microinjected into the wheat embryo sac on ovaries in situ but transformation was not achieved (Mathias 1987). Steinbiss et al. (1985) devised a strategy for transformation of barley by microinjection into the fertilized egg in isolated ovules and preliminary results for the cultivation of isolated ovules from immediately after fertilization were presented. Transformation of the egg cell should thus be possible by inserting the needle through the two surrounding integuments, the nucellus and into the egg cell. Töpfer and Steinbiss (1985) reported that it was possible to regenerate plants from isolated ovules of barley grown in liquid medium. 17% of the ovules, isolated 24 hours after fertilization gave rise to embryos and of these 20% germinated into plants. Plants were regenerated with a similar frequency from ovules isolated and cultured 90 min after pollination. It was documented that the regenerating plants originated from the fertilized egg by using genetic markers. Transformation of the fertilized egg was not reported.

Production of haploids or double haploids from microspores (Bajaj 1983, Dunwell 1985, Sangwan and Sangwan-Norreel 1987) or from unfertilized egg cells by cultivation of ovules (Dunwell 1985) are techniques already well established in the conventional breeding (Morrison and Evans 1988, Thomes 1990). A haploid plant which has undergone a spontaneous chromosome doubling or is induced to undergo this process will be homozygous for all its genes and directly give rise to a pure line following selfing. Since selfing over a number of generation is required in a conventional breeding program to establish a pure line, the double haploid technique has allowed for a reduction of the production time for new varieties.

In the grasses and in barley in particular (EP patent application n. 245 898, Hunter 1985, 1987, 1989, Lyne et al.1983, Foroughi-Wehr et al. 1976, Foroughi-Wehr og Fried 1984, Olsen 1987, 1991, Sunderland og Xu 1982) but also in wheat (Wei 1982, Datta og Wenzel 1987, Orchinsky et al.), rice (Cho and Zappata 1990, Datta et al. 1990) and maize (Coumans et al. 1989, Pescitelli et al. 1990) there are today well functioning techniques for the production of haploids and double haploids from immature pollen grains. Besides the potential for the production of double haploids for the conventional breeding, the techniques for the cultivation of immature pollen grains show great promise in other areas of

biotechnology, such as transformation and in vitro selection.

Efficient regeneration of plants from isolated microspores is, however, only possible in particular species and genotypes of these species (Sangwan and Sangwan-Norreel 1987). In these the microspores develop into embryos, that germinate into green fertile plants In other species and genotypes there is either no development of microspores or calli form instead of embryos. From calli the plant regeneration is less efficient and the regenerants often posses a number of genetic defects such as sterility or albinism, which results in the death of the plants at an early stage of development (Sunderland et al. 1985).

Regeneration of haploids by cultivation of the unfertilized egg in situ in the isolated ovule is another possibility for producing haploids and double haploids. In particular species such as sugar beet this technique functions very well, while f. ex in the grasses it is largely ineffective since only a few haploids have been reported to form (Dunwell 1985).

In summary it can be concluded, that the existing methods for regeneration of plants from protoplasts, cell aggregates or tissues in a large number of plants and in particular in our cereals are inadequate and very time consuming (see Potrykus 1991 and Morrish and Fromm 1992 for review). The establishment of the right type of suspensions is almost purely empirical where the right cell types are selected on the basis of size, color, density etc. The transformation and regeneration frequencies are low and during the often long lasting tissue culture steps, a number of genetic defects are induces which will appear as the so called somaclonal variation. The primary regenerants are usually partial or totally sterile and extensive breeding is required to remove these defects. It is also of major importance that plants only can be regenerated from suspension cells of a few genotypes. Finally, all transformation methods, including Agrobacterium mediated transformation, which have been used for the generation of transgenic plants of commercial interest, are based on the introduction of a selectable gene or a reporter gene. In biotechnology this may be of substantial importance, since legislation may not allow selectable genes in plants unless the objective is specifically to introduce herbicide resistance.

It is likewise reasonable to assume that the public opinion will be against the presence of foreign genes, conferring resistance to antibiotics/herbicides in transgenic plants used for animal and human feed. It may therefore be necessary to develop transformation methods, which allows for the removal of the introduced resistance gene at a later stage by cross breeding or other approaches.

In a number of animals transformation is today performed routinely by microinjection of genes in the male pronucleus of the fertilized egg. It is possible to obtain transformation frequencies of 25-50%. This implies that a selectable gene or a marker gene is not required and only the gene of interest is introduced. Microinjection is thus the only transformation technique available which circumvents the problem of selectable marker genes.

As described above it has been attempted to identify cell and tissue types from the higher plants and in particular from the cereals from which fertile plants can be regenerated and which can be manipulated using established techniques such as transformation, fusion and in vitro selection.

The fertilized egg cell in the higher plants is per definition totipotent, i.e., it has the ability to develop into an intact plant. The fertilized egg is therefore to be considered as suitable for transformation and in vitro selection and the unfertilized egg is the ideal cell for techniques such as in vitro fertilization with a sperm nucleus, protoplast fusion between two unfertilized egg protoplasts from the same or different species or genera or fusion between a fertilized egg protoplast and an enucleated egg protoplast (cybrid formation). Fusion of egg protoplasts thus allows for that the fusion product in contrast to the normally fertilized egg in most plant species contains cytoplasmic organelles such as plastids and mitochondria from both parents.

As described above it has not yet been possible to regenerate protoplasts of the fertilized egg or in vitro fertilized eggs. In maize it has been possible, by using cell wall degrading enzymes, to isolate unfertilized egg protoplasts and to electrofuse these with isolated sperm cells (Kranz et al 1991). The fusion products were cocultivated with a non-morphogenic maize cell suspension, 83% of the fusion products developed into microcalli, but these did not develop any further.

It is considered likely that the use of cell wall degrading enzymes can inhibit the subsequent regeneration process. Isolation of the egg protoplasts by cell wall degrading enzymes is moreover a very tedious and time consuming process since the egg protoplasts after the enzymatic digestion are to be identified and isolated from the mixture of other protoplasts and debris of the degraded ovules.

In barley anther culture conditioned medium has been used for supporting callus development from microspores in cultured anthers. The conditioned medium was obtained by culturing whole anthers or ovaries of barley in liquid medium, whereafter the medium was used for the cultivation of anthers for generating haploids and dihaploids from the anthers. Medium, previously used for the cultivation of anthers of oats, wheat, maize and tobacco also had a supporting effect on callus formation from anthers of barley (Xu et al. 1981). Sharp et al. (1972) cocultivated pollen grains of tomatoes with anthers of tomato where the pollen grains were placed on filter paper discs placed on top of anthers. Using this cocultivation technique, calli formed from up to 60 % of the pollen grains, while pollen grains grown in conventional medium did not respond with callus formation.

One have thus been aware of that anthers in some circumstances form or secrete compound which support callus formation from other anthers. The regeneration of fertile plants by this method has, however, not been reported.

In summary it is apparent that there is a great demand for the development of new techniques for the production of plants with special properties.

There is thus a special need to develop techniques for regeneration of intact fertile plants from cells and tissues which have undergone transformation, fusion, in vitro fertilization or other treatments. These techniques should allow for a fast, efficient and reproducible regeneration of basically all plant species.

This is in particular the case for plants belonging to the monocotyledonous plants, especially plants belonging to the family Gramineae, which include agronomically important culture plants such as wheat, barley, rye, oats, maize, rice, Sorghum and others which for these reasons are of particular economic importance.

There is also a great demand for the development of methods of isolation of egg protoplasts where these methods do not have an inhibitory effect on the subsequent regeneration process and which are simple and effective.

There is also a great demand for methods of transformation of egg cells where the egg cells subsequently can be regenerated by simple methods.

It is the aim of the present invention to provide methods which can meet the demands listed above.

In its first aspect the present invention relates to a method of regeneration of plant protoplast, cells or tissues to intact plants, a method which is simple, fast and efficient and in addition is remarkable as being universally applicable and by use of which method fertile green plants can be regenerated.

The method of regeneration of protoplasts, cells or tissues, of the type stated in the introductory part of claim 1 is characterized by the features defined in the characterizing portion of claim 1.

Protoplasts are defined as protoplasts from any plant tissues where their entire wall or parts of their wall are removed. They may be isolated from leaves, seeds, flowers, roots, pollen, embryos, ovaries or ovules or the protoplasts may be isolated from cell cultures including cultivated microspores, microspore derived embryos, secondary embryos from zygotic or microspore derived embryos or suspension cultures. It is common knowledge how to isolate protoplasts by enzymatic removal of the cell wall. Techniques for this are described in e.g. Potrykus and Shillito (1986) and in Kranz et al. (1991) and are also described in detail in DK Patent application n. 1099/89.

It is preferred to use protoplasts with a high regeneration potential such as protoplasts from meristems, embryos or egg cells. The latter are preferred since by using egg protoplasts one can obtain very high regeneration frequencies to healthy, fertile plants by using the procedure described in claim 1.

Cells are defined as single cells or cellular aggregates which also may be isolated from any tissue and parts of plants but are in particular suspension culture cells originating from meristems or embryos.

The cocultivation principle is well established in tissue culture, where protoplasts often are cocultivated with suspension cultures to support protoplast development (see Evans and Bravo 1983).

Cocultivation may involve a physical separation between the supporting culture and the cells to be supported and where it is ensured that low and high molecular compounds can diffuse between the two cultures. Typically, the protoplasts are embedded in a solid medium such as agarose while the supporting culture is in liquid medium overlaying the solid medium. For the regeneration of protoplasts, embryogenic as well as non-embryogenic suspensions have been used. In a recent study on regeneration of maize protoplasts (Petersen et al. 1992) protoplasts of cultures with different genetic backgrounds were cocultivated with embryogenic and non-embryogenic suspension cultures. It was concluded that regeneration only was efficient using different combinations of protoplast genotypes and suspension genotypes. If e.g. protoplasts of the line 21E were cocultivated with suspension cells of the same line, a

plating efficiency of 0.000015% was obtained, while cocultivation with suspensions of the line 203-12A resulted in a plating efficiency of 0.02%.

There is today very little evidence on the nature of the factors which during the cocultivation support regeneration and embryogenesis. In carrots a number of investigations have shown that during somatic embryogenesis specific extracellular proteins are present such as lipid transfer proteins (Sterk et al. 1991), peroxidases (Cordewener et al. 1991) or chitinases (De Jong et al. 1992). These are primarily to be regarded as markers for an embryogenic development, but it has also been shown that addition of some of these proteins can promote embryogenesis in cell cultures of carrot where the embryogenesis f.ex is inhibited by mutation. In barley comparisons between the proteins excreted from embryogenic and non-embryogenic cultures have also revealed considerable differences (Nielsen and Hansen 1992).

Microspore embryogenesis is a unique process in plants since it is the only tissue culture system available where a very large number of cells simultaneously undergo an embryogenic process.

It is thus this unique characteristic which is used in the method of regeneration of protoplasts, cells or tissues according to the invention. Microspores with their high regeneration potential contain a complex combination of compounds which support the regeneration of protoplasts, cells and tissues. This complex mixture of components may also be secreted/formed when the microspores undergo embryogenesis.

It is thus possible, if wanted, to use extracts of microspores or anthers, in particular microspores which are undergoing embryogenesis. Extracts are defined as non-intact cellular materials which are isolated from microspores or anthers, f.ex. by blending and possibly further purified by filtration or fractionation.

There are large differences between plant species and varieties as to the embryogenic capacity of microspores. Microspores from particular species and varieties thus possess a higher embryogenic potential than others.

Regeneration of so called dihaploid plants of barley from embryogenic, isolated microspores or microspores in cultivated anthers is today performed routinely. At the Carlsberg Research Laboratory (Olsen 1991) a microspore embryogenesis method has been developed, where the microspores are isolated by blending of anthers. The microspores are thereafter cultivated in so called Transwells (Transwell-COL, 24 mm, Costar #3418), immersed in culture medium. The semipermeable bottom of the Transwell ensures that the microspores are in good contact with the medium while an adequate supply of oxygen is provided since there is only a thin layer of medium covering the microspores. With this technique it is possible routinely to regenerate 10 green plants per anther using the barley variety Igri.

It is not possible to predict in a precise way whether a particular plant species or variety possess microspores with an optimal regeneration potential. Varieties and species with good microspore embryogenesis can only be identified in tissue culture experiments. A very larger number of investigations described in the literature, have however, analyzed a large number of species and varieties for their microspore embryogenesis potential.

In general, microspores or anthers from any species or variety may be used but the regeneration frequency of protoplasts, cells and tissues may depend on the source of microspores. The regeneration frequency also depends on the cell density of the microspores or the anthers cultivated in the medium.

Microspores or anthers from the barley cultivar Igri are particularly suitable for use in the method according to the invention.

Microspores or anthers and the protoplasts, cells and tissue to be regenerated can originate from the same species or variety or from different species or varieties.

Once a variety with highly embryogenic microspores has been identified, microspores and anthers from this variety can be used for the method described in the invention.

At least one microspore should be grown per cm^3 of medium before being used directly for cocultivation or for extraction of compounds to be used for supporting embryogenesis and regeneration. It is preferred to use microspore cultures at a density between $2x10^5$ and $3x10^5$ microspores per ml of culture medium or the equivalent amount of anthers. It is preferred, however, to use microspores.

Suspension cultures or callus cultures derived from microspores can also be used (Roberts-Oehlschlager et al. 1990). It is preferred though to use young cultures of microspores or anthers, i.e., preferably cultures that are younger than 30 days and in particular younger than 14 days. If the microspores or the anthers are not used for extracts it is preferred to use freshly isolated microspores and anthers or cultures of these which are only a few days old.

The microspores and the plant material to be regenerated can be cultured in the same medium which preferably is liquid or semi-liquid or the microspores can be cultivated in one medium and the plant material to be regenerated in a second medium which is in exchangeable contact with the first medium or contain fractions of the first medium. In the latter case the first medium is preferably liquid or semi-liquid while the second medium can be liquid, semi-liquid or solid.

Fractions are defined as dilutions of medium, in which there has been cultured microspores or anthers, where the dilution of the medium is with fresh medium or other compounds or where the medium used for culturing microspores and anthers is fractionated by physical or chemical methods such as a fractionation after molecular size, electric charge or other physical and chemical parameters. In general such a medium is composed of inorganic salts, amino acids, vitamins, hormones, carbohydrates and other defined organic compounds as well as more ill defined compounds such as extracts of plant tissue.

By using the method according to the invention it is thus possible to regenerate plant materials, which previously have been difficult to regenerate, to green fertile plants in much higher frequencies than seen before and it is also possible to regenerate plant materials, which previously have been impossible to regenerate.

It is of course obvious that the growth and culture media used for the tissue culture contain the nutrients essential for the plant material. The composition of nutrient in such growth and culture media are common knowledge to any person experienced in the field (se. f.ex. Kao and Michayluk 1975, Linsmaier et al. 1965, Spencer et al. 1990 and Twell et al. 1989).

If necessary the media may be further supplemented with growth promoting components.

The protoplasts, the cells or the tissue can before regeneration be manipulated and treated in various ways. They may f.ex. be transformed by the introduction of a DNA sequence in the host genome.

The cells and the protoplasts may have undergone chromosome doubling to form dihaploids or have been subjected to in vitro selection.

The protoplasts may have been subjected to fusion and egg protoplasts may be fertilized in vitro.

The present invention relates thus in its first aspect to additional methods for the generation of transgenic plants, hybrids and cybrids, homozygous plants and plants with special properties.

These methods are stated in claims 13, 14, 15 and 16.

By isolation of a protoplast, a cell or a tissue it is implied that the protoplasts, the cell or the tissue is present in a medium which basically is free of other tissue.

By using the methods stated in claims 13, 14, 15 and 16 it is thus possible to generate transgenic plants, hybrids, cybrids, homozygous plants and plants with special properties in a much simpler and faster way that by using the techniques previously known. It is furthermore possible by using the methods stated in claims 13, 14, 15 and 16 to generate transgenic plants, hybrids, cybrids, homozygous plants and plants with special properties which previously have been impossible to generate.

The invention concerns in addition in its first aspect the plants generated by these methods as well as regenerable parts of and progeny from these. This is stated in claims 17 and 18.

By regenerable parts are meant flowers, stems, fruits, seeds, leaves, roots, tissues from these parts, single cells and protoplasts as well as other parts which can be regenerated to plants.

The methods of the production of transgenic plants according to the first aspect of the invention comprises the following steps.

a) isolation of protoplasts, cells and tissues

b) introduction of foreign genetic material into the plant material described above in (a) or in the cases where the protoplasts are unfertilized egg protoplasts, in vitro fertilization with a sperm nucleus from another variety or species, preferably by microinjection, particle bombardment, electroporation, polyethylene glycol or laser and in particular by microinjection.

c) Regeneration of the transformed or in vitro fertilized protoplasts, cells or tissues by using the methods according to claims 1-12.

The protoplasts, the cells or the tissue can be isolated as normally done, i.e., mechanically or enzymatically.

The foreign genetic material can likewise be introduced using well known technology. For transformation by microinjection can be used equipment and procedures as described by Neuhaus et al. (1987), Lawrence og Davies (1985), Morikawa og Jamada (1985), Toyoda et al. (1987), Crossway et al. (1986) og Gordon and Ruddle (1983). As DNA solutions, to be used for the microinjections, can be used buffers with DNA in a suitable concentration. This DNA can be present as intact plasmid DNA, amplified and purified by conventional procedures (Maniatis et al. 1982), plasmid DNA linearized by cutting with a restriction enzyme in the plasmid sequence as well as genes (promoter-gene-terminator sequences), that are isolated and purified by conventional methods. Additional transformation methods to be

used are polyethylene glycol mediated transformation (Negrutiu et al. 1987), liposome mediated transfer (Gaboche 1990) or electroporation (Fromm et al 1987) or laser (Kaneko et al 1991) or other transformation techniques as described by Fraley et al. (1986) and Klein et al. 1988 and 1990.

The genetic material can be sense and antisense genes conferring resistance to antibiotics, herbicides, fungi, virus or insects, sense or antisense genes conferring male sterility, sense or anti sense genes affecting the quality of the plant as well as the quality of particular plants organs and plant products, including the production of new compounds in the plant.

The method of the production of hybrids and cybrids according to the primary aspect of the invention includes the following steps.

a) isolation of the first protoplast

b) isolation of a second protoplast or a sperm cell or if wanted the removal of the nucleus from the first or the second protoplast.

c) fusion of the two protoplasts f.ex. by treatment with polyethylene glycol or by electrofusion.

d) regeneration of the fused cells to hybrids or cybrids by using the method outlined in claims 1-12.

Isolated protoplasts of unfertilized eggs can be fused with isolated sperm cells as described by Kranz et al. (1991) or with other protoplasts of unfertilized eggs using protoplast fusion techniques (Evans 1983) using polyethylene glycol as described by Menczel and Wolfe (1984) or electrofusion, including microfusion between to individual protoplasts as described by Koop and Spangenberg (1989). The fusion products can thereafter be regenerated to normal fertile plants or hybrids with a high frequency using the method for cocultivation described above.

The steps described in steps a-c can be performed using the methods described above or as described in DK patent application no. 2632/87.

By using the method according to the first aspect of the invention for the production of homozygous plants the following steps are taken

a) isolation of haploid protoplasts or cells which may be induced to undergo chromosome doubling f.ex. by treatment with colchicine.

b) Regeneration of the double haploid protoplasts or cells to homozygous plants by using the method according to claims 1-12.

The events described in step a may be performed using the methods described in Bajaj 1983, Dunwell 1985, Sangwan and Sangwan Norreel 1987, Morrison and Evans 1988 and Thomes 1990.

By using the method according to the first aspect of the invention for production of plants with special properties, the following steps are followed.

a) isolation of protoplast or single cells

b) in vitro selection of the protoplast or single cells or cultures of these

c) regeneration of the protoplasts or single cells described in (b) to plants with the special properties by using the method according to claims 1-12.

In Handbook of Plant Cell Culture V, 1 (1. edition 1983-1986) are described various methods for in vitro selection, i.e., selection at the cellular level in cultures instead of selection on whole plants. In cell cultures it has thus been possible to select for f.ex. herbicide resistance or tolerance to stress such as low or high temperature, high salinity, drought etc.

The invention concerns in addition in its first aspect a medium to be used for the regeneration of protoplasts, cells and tissues.

The medium is presented in claim 19 and is characteristic by its content of extracts from microspores or anthers or components secreted from the microspores or the anthers during their cultivation, preferably to undergo microspore embryogenesis.

The selection of microspores or anthers, as well as the production of extracts are performed as described previously.

In it second aspect the present invention relates to a method of isolation of egg protoplasts from ovules, a method remarkable by being simpler and faster than previously known methods and which also maintains the ability of the egg protoplasts to give rise directly to a normal plants.

The method according to the invention for the isolation of egg protoplasts, which is of the type stated in the introductory portion of claim 20, is characterized by the features defined in the characterizing portion of claim 20.

Egg protoplasts isolated by the method of the invention can therefore with a high degree of probability, also after e.g. fusion, transformation or other manipulations be regenerated to healthy, green fertile plants.

The inventors of the present invention have thus regenerated more than 100 protoplasts of the fertilized egg to plants according to the method and have not yet observed the formation of any albino plant.

The method according to the invention for isolation of egg protoplasts, is in particular useful for isolation from angiosperms and in particular from monocotyledonous plants such as plants belonging to the Gramineae, including barley, wheat, Sorghum, rice and maize.

The egg protoplasts can be fertilized or unfertilized

The procedure according to the invention for isolation of egg protoplasts takes advantage of the fact that the vacuole of the central cell in the embryo sac has a higher turgor pressure than the surrounding tissue.

By puncturing the vacuole with a fine pointed forceps the sudden change in pressure will result in that the membrane of the egg is released from the wall, i.e., the egg is liberated as a spherical protoplast. The behavior of the egg cell is to some extent depending on the osmotic pressure of the medium.

The osmotic pressure of the medium thus has to be of an order of magnitude where the protoplasts neither is swelling to an extent that it ruptures or undergoes extensive shriveling due to dehydration.

Osmotic pressures ranging from 250 to 500 mOsm/kg are suitable, in particular a pressure of 375 mOsm/kg.

The osmotic pressure can be increased or reduced by addition/removal of maltose or the maltose may be partly replaced by mannitol.

A defined Kao medium is in particular well suited and additional calcium salts may be added.

The ovule resides within the ovary. The ovary may be completely removed or only the part surrounding the micropylar part of the ovule may be dissected away.

The results obtained reveal that by using the method according to claim 20 it is possible to isolate egg protoplasts from up to 80 % of the dissected ovules. It is possible for one person to isolate between 30 and 40 protoplasts per hour. The mechanical isolation according to the invention is thus much faster than the enzymatic procedure described earlier.

It is also possible to isolate protoplasts of the synergids by using the method of claim 20. The invention comprises thus in its first aspect also a method for the isolation of synergid protoplasts.

The method of the isolation of synergid protoplasts, which is of the type stated in the introductory portion of claim 21, is characterized by the features defined in the characterizing portion of claim 21.

The invention also includes the egg protoplasts and synergid protoplast isolated according to the methods in claims 20-26. This is stated in claim 27.

The isolated egg protoplasts as well as the isolated synergid protoplasts may be used in plant breeding in various ways.

The method of isolation of egg protoplasts according to claim 20 and the method of isolation of synergid protoplasts according to claim 21 can both result in that transgenic plants, hybrids or cybrids, homozygous plants and plants with special properties can be produced much faster and more efficiently than previously possible.

It is preferred to use isolated egg protoplasts since these cells have a greater potential for regeneration.

The present invention relates in its second aspect to further methods of production of transgenic plants, hybrids or cybrids, homozygous plants and plants with special properties.

These methods are stated in claims 28, 29, 30 and 31.

The invention includes also in its second aspect the plants generated by the methods as well as regenerable parts of these as well as their progeny. This is stated in claims 32 and 33.

The method for the production of transgenic plants according to the second aspect of the invention comprise the following steps.

a) Isolation of egg protoplasts according to claims 20 or claims 22-26.

b) The introduction of foreign genetic material in the egg protoplasts described in (a) and in the cases where the egg protoplasts are unfertilized , in the in vitro fertilized egg protoplasts using a sperm nucleus from a different variety or species, preferably by using microinjection, particle bombardment, electroporation, polyethylene glycol or laser and in particular by using microinjection.

c) Regeneration of the transformed or in vitro fertilized egg protoplasts.

In principle are all previously described transformation methods are useable in the method described above. It is preferred, however, to use the direct DNA transfer methods such as microinjection, particle bombardment, electroporation, polyethylene glycol or laser. These methods are described previously.

Regeneration of the transformed or in vitro fertilized egg protoplasts can take place in different ways. The egg protoplasts may e.g., be cultured in a medium containing the essential nutrients and as well as different regeneration promoting compounds. It is., however, preferred to regenerate the egg protoplasts by using the regeneration principle according to the first aspect of the invention.

To perform the methods of production of hybrids or cybrids according to the second aspect of the invention, the following steps are followed.

a) Isolation of the first egg protoplasts by using the methods of claims 20 or 22-26.

b) Isolation of a second protoplast or a sperm nucleus and if wanted the removal of the nucleus from the first or the second protoplast.

c) Fusion of the two protoplasts f.ex. by polyethylene glycol treatment or by electrofusion.

d) The regeneration of the fusion product to hybrids or cybrids where the methods of claims 1-12 may be used. The second protoplast may be an egg protoplast but can also be a protoplast from another material.

The methods for the production of cytoplasts (protoplasts without a nucleus) as well as for fusion are described previously, see f.ex. Evans (1983).

To perform the method according to the second aspect of the invention for the production of homozygous plants, the following steps are followed.

a) isolation of unfertilized protoplasts, which may be induced to undergo chromosome doubling f.ex. by treatment with colchicine.

b) Regeneration of the double haploid protoplasts or cells to homozygous plants by using the method according to claims 1-12.

To perform the method according to the second aspect of the invention for production of plants with special properties, the following steps are followed.

a) isolation of egg protoplast according to claim 20 or claims 22-26 whereafter the egg protoplasts may be further cultured.

b) in vitro selection of the protoplast or cultures derived from these.

c) regeneration of the selected protoplasts or cultures derived from these as described in (b) to whole plants with special properties preferably by using the methods according to claims 1-12.

The methods for in vitro selection have been described previously.

In its third aspect the invention relates to a method of transformation of fertilized egg cells or embryos smaller than 200μm in length. The method according to the third aspect of the invention is remarkable in that the transformed egg cells or embryos are able without additional treatment by e.g., hormones to regenerate into green fertile plants. Furthermore it is not necessary to introduce a selectable gene.

The invention which is of the type stated in the introductory portion of claim 34, is characterized by the features defined in the characterizing portion of claim 34

The microinjection methods and the apparatus to be used are described previously.

The method according to the third aspect of the invention is in particular useful for angiosperms and preferentially monocotyledons and especially plants belonging to the Gramineae. Plant with special commercial importance are barley, wheat, Sorghum, maize and rice and in particular barley and wheat.

The invention deals in its third aspect also with a method of production of transgenic plants. This is stated in claim 37.

The method of generation of transgenic plants according to the third aspect of the invention comprises the following steps.

a) Transformation in situ of egg cells or embryos by using the procedure according to claims 34-36.

b) Regeneration of the transformed egg cells or embryos by culturing whole ovules or the tips of ovules containing the transformed egg cells or embryos.

The invention also includes the transgenic plants and regenerable parts of plants from these as well as their progeny produced on the basis of the third aspect of the invention. This is stated in claims 38 and 39.

The invention is described in further details in the following text, the illustrations and the examples.

Figure 1 shows a cocultivation cell in cross section

Figure 2 illustrates a cross section of a barley ovary immediately after fertilization

Figure 3 is a histogram illustrating the fertility in spikes of barley plants grown from seeds and regenerated from egg cells by ovule culture.

Figure 4 is a histogram showing the number of spikes per plant from barley plants grown from seeds and regenerated from egg cells by ovule culture.

The cocultivation cell in Figure 1 consists of a dish and two inserts. The outer insert is a 24 mm Transwell (Costar #3418) and the outer a 12 mm Transwell (Costar #3405). The bottom of the two inserts consists of semipermeable membranes. The 24 mm Transwell is normally of the COL type with a pore size of the semipermeable membrane of 0.3μm while the 12 mm Transwell may be of either the col type or with a polycarbonate membrane with a pore size of 3μm.

About two ml of culture medium is added to the culture dish, which typically is a six well plate. In the dish is placed the 24 mm Transwell, containing the microspore culture as described in example 21. The protoplasts, cells or tissues to be regenerated are transferred to medium in the 12 mm Transwell, which is positioned inside the 24 mm Transwell.

The culture medium in the dish is a medium reservoir, where the medium components freely can diffuse through the semipermeable membrane of the 24 mm Transwell and thereby provide the microspore culture with a stable supply of fresh medium. The medium in the 24 mm Transwell can likewise freely diffuse through the semipermeable membrane of the 12 mm Transwell. Compounds secreted from the microspores can thus diffuse into the 12 mm Transwell containing the protoplasts, cells and tissues to be regenerated.

A cocultivation cell may of course be constructed in a number of other ways. It may be constructed as shown in Figure 1 but the microspores may be cultured directly in medium in the dish thereby avoiding the use of the first insert (the 24 mm Transwell). The microspores and the protoplasts, cells and tissues to be regenerated may also be present in

the same reservoir or dish, thereby avoiding the use of any of the two inserts.

The plants material to be regenerated may be imbedded in a solid medium such as agarose which is permeable for the components present in the microspore culture.

The only essential principle in the cocultivation is thus that the components present in the microspore culture undergoing embryogenesis readily can reach the material to be regenerated.

In the examples on cocultivation according to the first aspect of the invention the following cocultivation methods are used:

Method A. The cells to be regenerated are placed in a 12 mm Transwell insert (e.g. #3405, Costar) which subsequently is placed in a 24 mm Transwell insert (# 3418, Costar) containing the microspore preparation undergoing embryogenesis (see Fig. 1)

Method B. The microspore cultures from a 24 mm Transwell are transferred to one ml of medium and divided between two wells in a so called 12 well plate provided with the 12 mm Transwells. The cells to be regenerated are placed in a 12 mm Transwell which are placed in one of the microspore containing wells.

Method C. The cells to be regenerated are embedded in an agarose such as LMP agarose (LMP agarose, ultra pure, BRL 5517UB)(1.5% in Kao 90). The agarose beads containing the protoplasts or cells to be regenerated are thereafter cocultivated directly with the microspore preparation or transferred to a 12 mm or 24 mm Transwell and cocultivated as described in methods A and B.

The development of the female gametophyte, the embryo sac, takes place in basically the same manner in all the higher plants (see f.ex. Foster and Gifford 1959). The meiotic divisions give rise to a tetrad, and by subsequent divisions a haploid embryo sac is formed, consisting of one or two synergids, an egg cell, a central cell with one or more nuclei and

a number of antipodal cells. In most plants the so called double fertilization takes place, where a sperm nucleus fertilizes the egg, while the second sperm nucleus fuses with one or more central nuclei giving rise to the endosperm.

The composition of the ovary, the ovule and the embryo sac in barley immediately after fertilization is illustrated diagrammatically in Figure 2. The ovary possesses two stigmata. The ovule is located inside the ovary and consists of the embryo sac surrounded by the nucellus and the two integuments, referred to as the outer and the inner integument. The inner integument surrounds the embryo sac - nucellus completely while the outer integument is lacking in the micropylar region. The number of integuments varies between different plant species. The angiosperms normally possess two integuments while the gymnosperms lack integuments.

The embryo sac consists of a large central cell with two central or polar nuclei. At the chalazal end a number of antipodal cells are present while the micropylar part of the embryo sac contains two synergids and an egg cell. Two sperm nuclei are also indicated, one next to the nucleus of the egg and the other next to the two polar nuclei.

The structure of the embryo sac before, during and after fertilization has been described at the light and electron microscopical level by Mogensen (1982) and Engel (1989). In the barley variety Bomi, grown at 18°C, fertilization occurs 45 min after pollination. The pollen tube grows down along the integuments, enters the micropyle and into one of the synergids, which at this time has degenerated. The two sperm cells are extruded, one of the sperm nuclei enters the egg while the other sperm nucleus enters the central cell and fuses with the two central nuclei. This triploid nucleus will at a later stage give rise to the triploid endosperm. According to Engell (1989) the central nuclei moves away from the egg cell about 50 min after fertilization. Twenty hours after fertilization, the first divisions of the zygote is observed, after 22 hours 50% of the zygotes have divided once and after 24 hours all the zygotes have completed their first division.

The micropylar tip of the ovule can be exposed by removing the surrounding ovary tissue with a pair of fine tipped forceps. Inside the exposed ovule, the egg can only be identified

with some difficulty. It has on such specimens, as also analyzed by Töpfer and Steinbiss (1985) not been possible to perform a microinjection into the egg with sufficient precision. If, however, both the integuments are dissected away, it is possible in a dissection microscope and in particular in an inverted microscope such as a Zeiss Axiovert, equipped with differential interference contrast, to observe the egg cell at a high optical resolution. It is possible to see if the egg has been fertilized by using the movement of the fertilized central nuclei away from the egg as a marker for if fertilization has taken place, and the size and shape of the synergids serve as additional markers (see Engel 1989) for the developmental stage of the fertilized egg.

The unfertilized or fertilized egg can be isolated as a protoplasts by the following procedure: The micropylar tip of the ovary is exposed and the integuments are dissected away. Thereafter the large vacuole of the central cell is punctured by inserting a needle or one of the finely pointed legs of the forceps through the side of the ovule through the integuments and the nucellus tissue. Since the vacuole of the central cell possess a higher turgor pressure than the surrounding tissue, the sudden change in pressure causes the cell membrane of the egg to be released from wall of the egg cell. The dissected ovary is placed on a droplet of medium to stabilize for a minimum of 30 min. Thereafter the nucellus tissue of the micropyle is removed and the egg protoplast can be pushed out into the surrounding medium.

Often the persistent synergid is also pushed out as a protoplast, but it is easy to distinguish between the two protoplasts, since the protoplast of the egg is smaller and contains a granular cytoplasm with a centrally located nucleus, while the protoplast of the synergid has a smaller nucleus located at a peripheral position and a more homogenous cytoplasm. With a little practice it is possible to isolate egg protoplasts from about 80% of the dissected ovaries. About 30-40 egg protoplasts can be isolated per hour from dissected ovaries.

The protoplast of the egg can then be sucked up in a few μl of medium using f.ex. a Gilson pipette equipped with a sterile, cotton plugged pipette tip and transferred to further cultivation or manipulation such as microinjection or other transformation procedures. As other protoplasts, the egg protoplasts are very sticky and tend to bind to the surface of culture dishes etc. On should therefore use sterile dishes retreated for protoplast culture such

as Falcon Primaria #3802 (Beckton Dickinson), an agarose surface or a coverslip such as a 25x40 mm coverslip (Menzelgläser), which has not been cleaned by organic solvents or alcohol but only sterilized by autoclaving.

The egg protoplasts can be analyzed in detail using differential interference contrast optics on an inverted microscope, f.ex. a Zeiss Axiovert microscope. The optical resolution is sufficient to allow for that microinjection in the nucleus of these protoplasts can be performed with a sufficiently high optical resolution.

In Tables 1 and 2 are given the composition of the culture media used in the subsequent examples.

Table 1. Composition of the Kao 90 medium.

	mg/l
Macroelements:	
NH_4NO_3	165
NO_3	1900
$CaCL_2 \times 2H_2O$	440
$MgSO_4 \times 7H_2O$	370
KH_2PO_4	170
$FeNa_2$ EDTA	40
Microelements:	
H_3BO_3	6.2
$MnSO_4 \times 1H_2O$	16.9
$ZNSO_4 \times 4H_2O$	8.6
KI	0.83
$Na_2MoO_4 \times 2H_2O$	0.25
$CuSO_4 \times 5H_2O$	0.025
Organic elements	
BAP (benzylaminopurine)	1
Glutamine	750
Myo-inositol	100
Thiamin-HCl	1.0
Maltose	90,000
Nicotinamid	1.0
Pyridoxin HCl	1.0
D-Ca Panthothenate	1.0
Folic acid	0.4
p-ABA$^+$	0.02
Biotin	0.01
Cholin chloride	1.0
Riboflavine	0.2

Ascorbic acid	2.0
Vitamin A^+	0.01
Vitamin D_3^+	0.01
Vitamin $B12^+$	0.02
Na puruvate	5
Citric acid	10
Malic acid	10
Fumaric acid	10
Vitamin free casamino acids (Difco)	250
Coconut milk (Gibco nr. 570-5180)	20 ml

The vitamins were purchased as a ready to use mixture (Sigma K3129) and the remaining components added.

The medium was made up 2x concentrated, the pH adjusted to 5.8 using 1N NaOH and the solution sterilized by filtration (0.22μm pore size).

The Kao 60 and Kao 120 media only differ by the amount of maltose used (60g/l and 120 g/l)

Table 2. Composition of the LMS-60M medium.

Macroelements:	mg/l
NH_4NO_3	165
NO_3	1900
$CaCL_2$ x $2H_2O$	440
$MgSO_4$ x $7H_2O$	370
KH_2PO_4	170
$FeNa_2$ EDTA	40
Microelements:	
H_3BO_3	6.2
$MnSO_4$ x $1H_2O$	16.9
$ZNSO_4$ x $4H_2O$	8.6
KI	0.83
Na_2MoO_4 x $2H_2O$	0.25
$CuSO_4$ x $5H_2O$	0.025
Organic elements	
BAP (benzylaminopurine)	1
Glutamine	750
Myo-inositol	100
Thiamin-Hcl	0.4
Maltose	60,000

The medium was made up 2x concentrated, the pH adjusted to 5.8 using 1N NaOH and the solution sterilized by filtration (0.22μm pore size).

The same medium was used as regeneration medium but only contained 30 g/l maltose og 0.4 mg/l or no BAP. The medium was mixed with 0.8 % autoklaved SeaPlaque agarose.

Example1

Pollination, dissection of ovaries and cultivation of egg cells in situ in intact ovules.

Barley plants of the winter barley cultivar Igri or the spring barley cultivar Alexis were grown in growth cabinets or in a greenhouse equipped with heating/cooling facilities at 15°C/10°C day/night temperature using a 16 hours light period at a light intensity of ca 350 $\mu E \times m^{-2} \times s^{-1}$. The flowers were emasculated three to four days before flowering at a stage where the anthers still were green. When the stigmata were completely unfolded, a controlled pollination was performed using pollen from other Igri and Alexis plants.

The spikes were harvested 1-24 hours after pollination. The ovaries were carefully removed from the florets and placed on Kao90 medium. When all the ovaries of a spike had been isolated, they were transferred to 5 ml sterilization solution (10 % sodium hypochlorite BDH #23039 with 50 μl 10 % Tween 20 added) and sterilized for 10 min. After two washes in sterile water, the ovaries were transferred to Kao90 and dissected under a dissection microscope (40x magnification, transmitted light). The ovaries were placed with the vascular strand facing downwards and the stalk of the ovary and the two lodiculae were removed by using a fine tipped forceps. Thereafter a cross section was made with the tip of the forceps across the ovary at a median position and along the one side down to the micropylar end of the ovary. Only the ovary tissue was cut and care was taken not to cut into the wall of the ovule as well. Thereafter the tip of the ovary could be lifted away, exposing the micropylar end of the ovule. It is in such dissected ovaries possible to see if the central nuclei have moved away from the egg cell, thus illustrating if fertilization has occurred. The dissected ovaries were then transferred to 2 cm Whatman filter paper discs on agarose solidified medium: The ovaries were opened by cleaving them slightly to the one side of their longitudinal axis and the ovules lifted out and placed on the filter paper. The tip of the ovule containing the egg is normally bent away from the longitudinal axes of the ovule. The ovules were placed on the filter paper in such a way that the egg containing tip was facing away from the filter paper. During the removal of the ovule from the ovary, part of the outer integuments are often lost.

The culture dishes (Sterilin, 5 cm Bibby Sterilin, U.K.) were sealed with Nesco film (Nippon Shoji Kaisha, Japan) and wrapped in alu foil and incubated at 23°C. After three weeks, the dishes were opened and the developing embryos removed from the remains of the ovule. In some cases more than one embryo had developed within each ovule, in this case the embryos were separated from each other to allow for the germination of more than one plant.

When the embryos after three to four weeks started to germinate, they were transferred to regeneration medium in Sterilin dishes and cultured under low light conditions at 23°C. When additional growth had occurred, the plantlets were moved to fresh regeneration medium in 10 cm high cylindrical containers (Greiner, Bibby Sterilin, U.K.). For the winter barley Igri, the plantlets were transferred to 4°C for eight weeks for vernalisation when large plantlets had formed, filling the container. Thereafter the plantlets were transferred to soil and cultured in a greenhouse (15/10 °C, 16 hours light period). Plantlets of the spring barley Alexis were not vernalized.

Example 2

Effect of different media and hormones on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of four spikes of **Igri** were isolated as described in example 1, **8** hours after pollination and cultured on seven different agarose solidified media.

M1: Kao 90
M 2: Kao 90 without caseinhydrolysat
M 3: Kao 90 without coconut water
M 4: Kao 90 without BAP
M 5: Kao 90 without BAP but with 1 mg/l 2,4-D (2,4 dichloro phenoxy acetic acid)
M 6: Kao 90 without BAP but with 2 mg/l 2,4-D
M 7: Modified MS with 60 g/l maltose

The embryos were dissected out of the remains of the ovule after 27 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No. of ovules	No. of ovules with embryo formation	No. of plants after 33 days	No. of plants after 41days	No. of plants after 58 days
M 1	10	9	11	11	14
M 2	10	7	5	9	9
M 3	10	9	6	8	8
M 4	10	5	1	3	3
M 5	10	8	0	10	14
M 6	10	8	2	9	10
M 7	10	8	13	14	14

Conclusion: The fastest growth and the best embryo formation was obtained on Kao 90 and on LMS-60 medium. Kao90 without BAP resulted in a very slow embryo development. Kao 90 without BAP but with 1 or 2 mg/l 2,4-D resulted in the formation of very compact callus like structures, that germinated into plantlets at a much later stage than when cultured on the normal Kao90 medium. The lack of casein hydrolysate or coconut water also resulted in a slow growth of the embryos.

Example 3

Effect of different media and hormones on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of four spikes of **Igri** were isolated as described in example 1, **8** hours after pollination and cultured on five different agarose solidified media.

M 1: Kao 90
M 2: Kao 90 with 1 mg/l 2,4-D
M 3: Kao 90 without BAP
M 4: Kao 90 without BAP but with 1 mg/l 2,4-D
M 5: Kao 90 without BAP but with 2 mg/l 2,4-D

The embryos were dissected out of the remains of the ovule after 27 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No. of ovules	No. of ovules with embryo formation	No. of plants after 35 days	No. of plants after 45 days	No. of plants after 53 days
M 1	12	11	10	10	10
M 2	13	5	4	5	5
M 3	14	8	2	4	4
M 4	13	13	2	12	14
M 5	12	9	3	8	8

Conclusion: The same development was observed on the different media as seen in example 2. Kao 90 with 1 mg/l 2,4-D gave the poorest embryo formation with malformed and miscolored embryos.

Example 4

Effect of different media and hormones on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of four spikes of **Igri** were isolated as described in example 1 **8**, hours after pollination and cultured on seven different agarose solidified media.

M1: Kao 90
M 2: Kao 90 without caseinhydrolysat
M 3: Kao 90 without coconut water
M 4: Kao 90 without BAP
M 5: Kao 90 without BAP but with 1 mg/l 2,4-D
M 6: Kao 90 without BAP but with 2 mg/l 2,4-D
M 7: Kao 90 with 1 mg/l 2,4-D

The embryos were dissected out of the remains of the ovule after 27 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No. of ovules	No. of ovules with embryo formation	No. of plants after 27 days	No. of plants after 35 days	No. of plants after 42 days
M 1	7	7	3	6	7
M 2	6	4	1	2	2
M 3	6	5	4	4	4
M 4	6	4	0	0	0
M 5	6	4	0	0	0
M 6	6	5	0	3	3
M 7	6	4	0	2	2

Conclusion: The same development was observed as seen in examples 2 and 3

Example 5

Effect of different media and hormones on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of one spike of **Igri** were isolated as described in example 1, **24** hours after pollination and cultured on four different agarose solidified media.

M 1: Kao 90

M 2: Kao 90 without BAP

M 3: Kao 90 without BAP but with 1 mg/l 2,4-D

M 4: Kao 90 with 1 mg/l 2,4-D

The embryos were dissected out of the remains of the ovule after 27 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No. of ovules	No. of ovules with embryo formation	No. of plants after 35 days	No. of plants after 49 days
M 1	5	5	2	3
M 2	5	4	0	2
M 3	5	5	0	3
M 4	5	5	3	3

Conclusion: The same development was observed as seen in examples 2 and 3

Example 6

Effect of different media and hormones on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of four spikes of **Alexis** were isolated as described in example 1, **8** hours after pollination and cultured on six different agarose solidified media.

M1: Kao 90
M 2: Kao 120
M 3: Kao 60
M 4: Kao 90 without BAP
M 5: Kao 90 without BAP but with 2 mg/l 2,4-D
M 6: Kao 90 with 1 mg/l 2,4-D

The embryos were dissected out of the remains of the ovule after 27 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No.of ovules	No. of ovules with embryo formation	No. of plants after 22 days	No. of plants after 32 days	No. of plants after 54 days
M 1	10	7	3	8	9
M 2	10	10	3	20	20
M 3	10	7	4	11	12
M 4	10	9	0	1	4
M 5	10	6	0	0	3
M 6	10	6	0	0	5

The same development was observed for Alexis as observed for Igri in examples 2-4.

Example 7

Effect of different media and hormones on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of one spike of **Alexis** were isolated as described in example 1, 8 hours after pollination and cultured on two different agarose solidified media.

M 1: Kao 90

M 2: Kao 90 med 1 mg/l 2,4-D

The embryos were dissected out of the remains of the ovule after 34 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No. of ovules	No. of ovules with embryo formation	No. of plants after 24 days	No. of plants after 34 days	No. of plants after 49 days
M 1	10	6	6	6	7
M 2	10	8	0	2	5

Example 8

Effect of different media and hormones on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of one spike of **Alexis** were isolated as described in example 1, **24** hours after pollination and cultured on two different agarose solidified media.

M 1: Kao 90

M 2: Kao 90 med 1 mg/l 2,4-D

The embryos were dissected out of the remains of the ovule after 34 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No. of ovules	No. of ovules with embryo formation	No. of plants after 33 days	No. of plants after 48 days	No. of plants after 55 days
M 1	11	9	10	10	11
M 2	11	10	0	0	2

Example 9

The effect of removing the outer or both integuments from the micropylar end of the ovule on the regeneration of plants by in situ culture of fertilized eggs in intact ovules.

Ovules of one spike of **Igri** were isolated as described in example 1, 3 hours after pollination and cultured on Kao 90-agarose medium.

M 1: Both integuments were removed from the micropylar part

M 2: Only the outer integument was removed

The embryos were dissected out of the remains of the ovule after 34 days and after germination transferred to MS regeneration medium and cultured under the ordinary circumstances.

	No. of ovules	No.of ovules with embryo formation	No. of plants after 33 days	No. of plants after 40 days	No. of plants after 47days
M 1	11	10	7	10	14
M 2	9	7	6	6	6

Conclusion: It was apparent that embryo formation actually was faster in ovules, where both integuments had been removed from the micropylar part than in ovules where only the outer integument was removed.

Example 10.

Cultivation of fertilized eggs and small embryos in isolated tips of ovules.

Ovaries from spikelets of the varieties **Igri** og **Alexis** were isolated from one hour to one week after fertilization. The length of the ovary was measured as an indication of the developmental stage of the embryo. Ovaries of 2 mm in length thus contain fertilized eggs while ovaries of a length of 7-8 mm contain embryos about 200 μm i length. The ovaries were sterilized and dissected as described in example 1, the tip of the ovule, containing the egg-embryo, cut off and transferred to LMS-90 with 20% Ficoll 400 (Pharmacia). A 20 % Ficoll solution gives the medium sufficient density to allow for that the tips of the ovules can float on the surface of the LMS-Ficoll medium.

	ALEXIS		IGRI	
	Tips of ovules		Tips of ovules	
Ovary length	No. of ovule tips cultivated	No.of ovule tips with embryo formation %	No. of ovule tips cultivated	No.of ovule tips with embryo formation %
7-8 mm	15	100	12	92
6-7 mm	18	83	9	56
5-6 mm	24	88	8	50
4-5 mm	16	63	10	70
3-4 mm	27	19	13	23
2-3 mm	22	18	12	25
2 mm	22	23	23	30

Conclusion: Embryos from ovules which have reached a length of 7-8 mm have started to differentiate the tissues characteristic for the mature embryo, such as apical meristems and scutellum and can survive as isolated structures. For Igri as well as Alexis it is apparent that 20-30 % of the fertilized eggs and small embryos can develop into embryos.

Example 11.

Cultivation of fertilized eggs and small embryos in isolated tips of ovules.

Ovaries from spikelets of the variety **Igri** were isolated from one hour to one week after fertilization. The length of the ovary was measured as an indication of the developmental stage of the embryo. Ovaries of 2 mm in length thus contain fertilized eggs while ovaries of a length of 7-8 mm contain embryos about 200 μm i length. The ovaries were sterilized and dissected as described in example 1, the tip of the ovule, containing the egg-embryo far cut off and transferred to LMS-90 with 20% Ficoll 400 (Pharmacia) or solid Kao90-agarose medium. A 20 % Ficoll solution gives the medium sufficient density to allow for that the tips of the ovules can float on the surface of the LMS-Ficoll medium.

	20 % Ficoll		0.8 % agarose	
	Tips of ovules		Tips of ovules	
Ovary length	No. of ovule tips cultivated	No.of ovule tips with embryo formation %	No. of ovule tips cultivated	No.of ovule tips with embryo formation %
4.5-5.0 mm	10	60	ND	ND
4.0-4.5 mm	27	33	23	83
3.5-4.0 mm	29	14	ND	ND
3.0-3.5 mm	16	25	22	91
2.5-3.0 mm	14	29	ND	ND
2-2.5 mm	22	18	ND	ND
2 mm	22	23	65	30

Conclusion: On both media approximately the same number of structures develop. There is, however, a qualitative difference as the embryos developing on the Kao90 medium, in particular the solid medium, are more embryo like than the structures developing in the LMS-60 liquid medium.

Example 12

Cultivation of unpollinated ovules.

Ovaries from 10 spikes of **Igri**, ranging in development from two weeks before flowering until immediately before flowering were isolated, sterilized and the ovules dissected out as described in example 1. The ovules were thereafter cultured on Kao90-agarose as also described in example 1. In none of the cultured ovules were there any indication of embryo formation even after several weeks of culture.

Example 13

Destruction of the fertilized egg cell by puncturing with a bevelled injection needle.

Forty ovaries from two spikes of **Igri** were isolated and sterilized as described in example 1, 8 hours after pollination. The integuments were removed from the tip of the ovules and the dissected ovaries mounted for microinjection as described in example 15.

The needles for microinjection were prepared as described in example 15 and the tip thereafter bevelled into the shape of a hypodermic needle using a Narishige microgrinder. It is with this kind of needle possible to puncture and destroy the egg cell with a high degree of precision without affecting the persistent synergid.

The ovules with the destroyed egg cells were thereafter cultured as described in example 15. After several weeks of culture there were no indications of embryo formation in the ovules illustrating that the embryos form from the egg and not from any other tissues or cells in the ovule.

Example 14

Number of spikes and fertility of plants of the variety Igri regenerated from culturing fertilized egg cells in intact ovules.

The plantlets originating from the cultivation of fertilized egg cells in ovules were cultured and vernalized as described in example 1. The plants were transferred to soil and grown in the greenhouse until maturity. At this stage the number of spikes on each plant as well as the degree of fertility was measured and compared to similar data obtained from control plants, grown from seeds in the same green house under the same

conditions. The data presented in Figures 3 and 4 shows that the Igri plants regenerated in ovule culture possess about three times as many spikes per plant as the seed grown control material. This can in part be attributed to the fact that these plants prior to vernalization were cut back to facilitate the production of more tillers but can also be attributed to the presence of more apical meristems in the embryos obtained from the ovule cultures. There is a slightly higher fertility in the seed grown material, a result which also may relate to the difference in the number of spikes since the last forming spikes on the plants grown from fertilized eggs in ovules often were partly to completely sterile.

Example 15.

Microinjection into the fertilized egg cell in ovules where the outer integument had been removed from the micropylar region.

Ovaries from Igri and Alexis were isolated, sterilized and dissected as described in example 1 and the outer integument was removed from the micropylar part of the ovule. The ovaries with the exposed tip of the ovules were thereafter mounted for microinjection in the following way: The outermost 2-3 mm of the sides of a 24 x 40 mm cover slip was covered with a rim of silicone glue (Fugendichtung silicon, Practicus Chemie, Germany) and the cover slips were thereafter sterilized by placing them under uv illumination for a minimum of 30 min. The ovaries were mounted in the silicone glue in such a way that the tips of the ovules were free of the glue and faced towards the center of the coverslip. It was also essential for the subsequent optical analysis that the longitudinal axes through the egg cell was parallel with the surface of the cover slip. Kao 90 medium was added so that the ovule tip was just covered with medium.

The ovule tips and the eggs were analyzed under differential interference contrast on an Axiovert microscope (Zeiss) and the microinjection performed and monitored using a high magnification and a TV screen (objective: x40; ocular: x10, TV tube magnification: x4). The injection needles were either pulled from borosilicate micro capillaries (Micro electrode capillaries, 1.5 mm in outer diameter, Hilgenberg, Germany) using a vertical puller (Bachofer, Germany) or ready made injection needles (Eppendorf femtotips) were used.

The injection solution was plasmid DNA or isolated genes in a concentration of 1-100 ng/μl in 10 mM Tris + 0.1 mM EDTA. The gene encoding for β-glucuronidase (GUS) was used for most experiments under the control of either the 35S promoter with the insertion of the intron 1 from the alcohol dehydrogenase 1 of maize (Callis et al. 1991). In both cases the NOS sequence was used as the terminator. These constructs provide for a high constitutive expression of the GUS gene. The activity of the GUS enzyme can be measured by using a histochemical assay, where the GUS enzyme cleaves the so called x-Gluc substrate. By an oxidative dimerization of the liberated indolyl compound the blue dye indigo is formed. Alternatively a fluorometric assay is used where the substrate is methyl umbelliferon glucuronide. The methyl umbelliferon can thereafter be measured fluorometrically (see Scott et al. 1988).

The DNA solution was sterilized by filtration through a filter with a pore size of 0.45μm and centrifuged for 30 min at 17,000 x g. The solution was transferred to the injection needles by using an Eppendorf Microloader. About one μl was transferred to each needle.

The injection was performed using an Märtzhauser micomanipulator and an Eppendorf Microinjector #5242 as described by Ansorge (1982). The needle was inserted though the inner integument and the nucellus tissue into the egg cell and the injection monitored by observing the displacement of cytoplasmic and nuclear material by the microinjection fluid extruded.

After injection the ovules were transferred to Kao90-agarose medium and the ovules dissected out and cultured as described in example 1.

The histochemical assay for GUS was performed as described by Scott et al. (1988) on whole ovules or tips of ovules two to three days after injection. Transient expression of the introduced gene was observed in the central cell as well as the egg cell/embryo.

Example 16

Cultivation of fertilized eggs in ovules where the outer integument had been removed and where DNA had been microinjected.

Ovaries of **Igri** were isolated **1-24** hours after pollination, sterilized and dissected to expose the tip of the ovule as described in example 1. The outer integument was removed from the micropylar part of the ovule. The eggs were microinjected with different DNA constructs (GUS, CAT: the gene encoding for chloramphenicol acetyltransferase; BAR: the gene encoding for phosphinothricin acetyltransferase) dissolved in 10 mM Tris + 0.1 mM EDTA, pH 7.5.

Frequency of embryo formation and the number of regenerated plants from non-injected and injected egg cells in ovules of Igri.

Material	Variety	Injected gene	No. of ovules cultured	No. of embryos formed	Frequency of embryo formation %	No.of plants regenerated
Injected	Igri	EMU-GUS	338	149	44	193
Injected	Igri	35S-BAR 35S-CAT	160	76	48	147
Control	Igri	-	115	93	81	46

Conclusion: Microinjection results in that only half the embryos are formed. There are, however, more embryos forming per egg cell in the microinjected material.

Example 17

Cultivation of fertilized eggs in ovules where both integuments have been removed and where DNA has been microinjected.

Ovaries of **Igri** were isolated **1-24** hours after pollination, sterilized and dissected to expose the tip of the ovule as described in example 1. Both integuments were removed from the micropylar part of the ovule. The eggs were microinjected with the isolated gene sequence for EMU-GUS-NOS in concentrations of 1-25 ng/μl in 10 mM Tris + 0.1 mM EDTA.

Frequency of embryo formation and the number of regenerated plants from egg cells of Igri injected with the EMU-GUS-NOS sequence.

Variety	Injected gene	No. of ovules injected	No. of ovules with embryo formation	No. of embryos formed	No. of plants regenerated
Igri	EMU-GUS	101	33	98	43

Example 18

Ovaries of three spikes of the spring wheat variety Ciano and three spikes of the spring wheat variety Walter were isolated 20 hours after pollination and sterilized as described for barley in example 1. The ovaries were dissected as described for barley and cultured on agarose-Kao 90 medium. Embryo formation was observed in eight percent of the cultured ovules. These were transferred to regeneration medium as described for barley in example 1. The embryogenic structures germinated to normal plantlets and were transferred to soil and grown in a growth cabinet at 15/10°C day/night temperature with a 16 hours light period. The plantlets develop into completely normal wheat plants. On each plant was formed about 30 spikes, all 100 % fertile.

Example 19

Mechanical isolation of protoplasts of fertilized eggs of barley and cultivation in liquid Kao90 medium or solid Kao90-agarose medium.

Ovaries of two spikes of **Igri** (**8** and **16** hours after pollination) were isolated and sterilized as described in example 1. Both integument were removed from the micropylar part of the ovule and the vacuole in the large central cell of the embryo sac punctured by inserting the fine pointed one leg of a pair of forceps through the nucellus tissue. The ovaries were placed on droplets of Kao 90 for a minimum of 30 min to allow for a stabilization of the egg protoplast. Thereafter the micropylar part of the nucellus tissue was removed and the protoplast of the fertilized egg gently pushed out. The protoplast was isolated in 3 μl Kao 90 and transferred to a droplet (15μl) of 1.5% LMP agarose in Kao90 on a sterile cover slip. The agarose-Kao90 solution was maintained at a temperature of 35-40°C before being used for the embedding of the protoplasts. When the agarose bead had solidified a few μl of additional LMP agarose-Kao90 was added to the top of the agarose drop.The cover slip with the agarose beads was placed in a Falcon Primaria Organ Tissue Culture Dish #3037 and sealed with Nescofilm. Water was added to the outer chamber to prevent desiccation of the cultures.

The development of the protoplasts was monitored by microscopical observations over a period of 14 days. All cells in a preparation of 40 protoplasts divided a couple of times, whereafter their growth ceased and the structures degenerated.

Ovaries of two spikes of **Igri** (**8** and **16** hours after pollination) were isolated and sterilized as described in example 1. Both integument were removed from the micropylar part of the ovule and the vacuole in the large central cell of the embryo sac punctured by inserting the fine pointed one leg of pair of forceps through the nucellus tissue. The ovaries were placed on droplets of Kao 90 for a minimum of 30 min to allow for a stabilization of the egg protoplast. Thereafter the micropylar part of the nucellus tissue was removed and the protoplast of the fertilized egg gently pushed out. The protoplast was isolated in 3 μl Kao 90 and transferred to 1 ml of Kao90 on a sterile uncleaned cover slips which was placed in a Falcon Primaria Organ Tissue Culture Dish #3037 and sealed with Nescofilm. Water was added to the outer chamber to prevent desiccation of the cultures.

The development of the protoplasts was monitored by microscopical observations over a period of 14 days. All cells in a preparation of 40 protoplasts divided a couple of times whereafter their growth ceased and the structures degenerated.

Example 20.

Microinjection into protoplasts of the fertilized egg

For the microinjection into the protoplast of the fertilized egg was used the same equipment as described in examples 15 and 16. The protoplasts were either embedded in agarose as described in example 19 or immobilized by using a so called holding capillary, where the protoplast is held in place at the end of a glass capillary by applying a slight negative pressure to the capillary. The edges of the capillary were melted by using a so called micro forge (Research Instruments), where the rim of the glass is melted by using a heated filament

Under differential interference contrast and a high magnification it is possible to perform a precise and controlled microinjection into the nucleus of the fertilized egg. It is preferred to microinject the egg as early as possible after pollination since the DNA replication of the fertilized egg not yet has commenced (se Friedman 1991 for a survey of data on DNA replication in the fertilized egg). If the DNA is injected after the DNA replication has taken place the introduced gene will only be present in one of the two chromatids and already after the first division of the zygote a chimeric structure is established, i.e., only one of the two daughter cells possess the introduced gene.

After microinjection the egg protoplasts are transferred to cocultivation with microspores by using a pipette or if they were embedded into agarose, an agarose cube containing the egg was cut out and transferred to cocultivation.

Example 21

Isolation and cultivation of microspores.

A detailed description of the isolation and cultivation of microspores are given in Olsen 1987 and 1991. Barley plants of the variety Igri are grown in growth cabinets or green houses at 15°C/10°C day/night temperatures with a 16 hours light period. The spikes are isolated when the spikes have reached the late uninuclear stage, the anthers are taken out and placed on 0.3 M mannitol for three days at 25°C in the darkness. Alternatively the spikes are isolated when the microspores have reached the mid uninuclear stage and placed at 4°C for 28 days. The microspores are isolated from the anthers by microblending in 15 ml 0.3 M mannitol in a microblender consisting of a Waring motor #8420 and an Eberbach rotor # 8575 for 30 seconds. The microblended suspension is filtered through a 100μm nylon filter and centrifuged for 5 min at 50 x g in a swing out rotor. The supernatant is removed and the microspores resuspended in 10 ml LMS-60M. After an additional centrifugation the microspores are resuspended in 5 ml LMS-60M or Kao 60. A sample of the microspore preparation is counted in a Burker-Türk hemocytometer and the preparation adjusted to a density of 2-3 x 10^3 microspores per ml.

The microspore preparation is transferred to 24 mm Transwell inserts (#3418, Costar, one ml per insert) which are placed in Costar "cluster plate wells" in 2 ml LMS-60M. The Costar plates are sealed with Nescofilm and incubated at 25°C in the darkness.

In these cultures typically 20-30 % of the microspores are of an embryogenic type, that starts to divide. After 10-12 days the microspore wall is ruptured and over the next 14 days bipolar, embryo like structures form from the embryogenic microspores. After four weeks the embryos can be transferred to solid medium (0.8% Sea Plaque agarose) containing LMS medium with 0.4 mg/l BAP and 30 g/l maltose and start to germinate into plantlets. Typically 10 green plants can be regenerated from the microspores of one anther.

Example 22.

Barley plants of the variety Igri were grown in growth cabinets and the greenhouse at 15°C/10°C day/night temperature with a 16 hours light period. The spikes were isolated when the spikes had reached the late uninucleate stage, the anthers are taken out and placed on 0.3 M mannitol for three days at 25°C in the darkness. Alternatively the spikes are isolated when the microspores have reached the mid uninuclear stage and placed at 4°C for 28 days. The anthers were taken out and either placed on solid LMS-60M medium with o.8% agarose or on liquid LMS-60M medium with 20% Ficoll. The dishes were sealed with Nescofilm and incubated at 25°C in the darkness.

After three weeks the anther locules burst because of the formation of embryos from microspores. The embryoids are harvested and transferred to regeneration medium as described for the cultivation of isolated microspores.

Example 23

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture: The effect of cocultivation technique.

Protoplasts of the fertilized egg were isolated from two spikes of **Igri, 7** hours after pollination as described in examples 1 and 18 and thereafter cocultivated with an embryogenic microspore preparation.

M1: Fourteen protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a 10 day old microspore culture grown in a 24 mm Transwell (cocultivation technique A).

M2: The protoplasts were embedded in 1.5% LMP agarose in Kao 90. The following day 8 protoplasts were transferred to a 12 mm Transwell and cocultivated with an 11 day old microspore culture grown in a 24 mm Transwell (cocultivation technique A).

	Cultivation method	No. of protoplasts	No. of developing structures after 21 days	No. of embryogenic structures after 42 days
M 1	Direct cultivation	14	14	8
M 2	Embedded in agarose	8	4	1

Conclusion: The protoplast regeneration appears to be more effective when using a direct culture of non-embedded protoplasts.

Example 24

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture: The effect of the age of the microspore culture.

Protoplasts of the fertilized egg were isolated from three spikes of **Igri, 7 hours** after pollination as described in examples 1 and 18 and thereafter cocultivated with an embryogenic microspore preparation.

M1: Fourteen protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a freshly isolated microspore culture grown in a 24 mm Transwell (cocultivation technique A).

M2: Nine protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a 17 days old microspore culture grown in a 24 mm Transwell (cocultivation technique A).

M3: Nine protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a 27 days old microspore culture grown in a 24 mm Transwell (cocultivation technique A).

	Age of the microspore culture	No. of. protoplasts	No. of developing structures after 14 days
M 1	0	14	7
M 2	17	9	0
M 2	27	9	0

Conclusion: Microspore cultures 17 and 27 days old do not support protoplast regeneration in the variety. This may either be attributed to that these cultures, which by now are growing rapidly, depletes the medium for all its nutrients or alternatively that the embryogenic structures at this stage no longer secretes compounds essential for the protoplast regeneration.

Example 25

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture: The effect of the age of the microspore culture.

Protoplasts of the fertilized egg were isolated from three spikes of **Igri, 7 hours** after pollination as described in examples 1 and 18 and thereafter cocultivated with an embryogenic microspore preparation.

M1: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a 6 days old microspore culture grown in a 24 mm Transwell (cocultivation technique A).

M2: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a 22 days old microspore culture grown in a 24 mm Transwell (cocultivation technique A).

	Age of the microspore culture	No. of. protoplasts	No. of developing structures after 14 days
M 1	6	10	3
M 2	22	10	0

Conclusion: Microspore cultures 22 days old do not support protoplast regeneration in the variety Igri. This may either be attributed to that these cultures, which by now are growing rapidly, depletes the medium for all its nutrients, or alternatively that the embryogenic structures at this stage no longer secretes compounds essential for the protoplast regeneration.

Example 26

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture: The effect of different cocultivation techniques.

Protoplasts of the fertilized egg were isolated from three spikes of **Igri**, 7 hours after pollination as described in examples 1 and 18 and thereafter cocultivated with an embryogenic microspore preparation.

M1: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with an 8 days old microspore culture grown in a 24 mm Transwell (cocultivation technique A).

M2: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with an 8 days old microspore culture which after seven days of culture in a 24 mm Transwell was divided between two wells in a 12 well plate (cocultivation technique B).

	Cocultivation method	No. of protoplasts	No. of developing structures after 21days	No. of plants after 38 days	No. of plants after 60 days
M 1	A	10	5	0	0
M 2	B	10	8	4	7

Conclusion: There appears to be a better protoplast regeneration using cocultivation method B. This may be attributed to the fact that the 12 mm Transwell insert rest on the rim of the well in the 12 well plate and not on the bottom of the well, whereas the 12 mm Transwell insert rests directly on the bottom of the 24 mm Transwell in cocultivation technique A. In technique B there may thus be a better possibility for the free diffusion of compounds secreted from the microspores into the Transwell insert containing the protoplasts.

Example 27

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture: The effect of different cocultivation versus cultivation in medium only.

Protoplasts of the fertilized egg were isolated from two spikes of **Igri, 18** hours after pollination as described in examples 1 and 18.

M1: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a 6 days old microspore culture transferred to a 12 well plate (cocultivation technique B).

M2: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cultured in Kao 90 in a 12 well plate.

M3: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cultured in Kao 90 + 1 mg/l 2,4- D in a 12 well plate.

	Culture method	No. of protoplasts	No. of developing structures after 14 days	No of plants after 53 days
M 1	6 days old microspore culture	10	7	11
M 2	Kao 90	10	0	0
M 2	Kao 90 + 1 mg/l 2,4-D	10	0	0

Conclusion: Protoplasts of the fertilized egg cannot be regenerated by culture in medium such as Kao90 or Kao90+ 2,4-D. Only by cocultivation is it possible to regenerate larger structures and plants.

Example 28

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture: The effect of cocultivation versus cultivation in medium only.

Protoplasts of the fertilized egg were isolated from two spikes of **Igri, 5** hours after pollination as described in examples 1 and 18.

M1: Ten protoplasts were transferred in Kao 60 to a 12 mm Transwell and cocultivated with a 6 days old microspore culture transferred to a 12 well plate (cocultivation technique B).

M2: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cultured in Kao 90 + 1 mg/l 2,4- in a 12 well plate.

	Culture method	No. of protoplasts	No. of developing structures after 14 days	No. of plants after 51 days
M 1	Kao 60 + a 6 days old microspore culture	10	8	2
M 2	Kao 90 + 1 mg/l 2,4-D	10	0	0

Conclusion: Protoplasts of the fertilized egg cannot be regenerated by culture in medium such as Kao90 + 2,4-D. Only by cocultivation is it possible to regenerate larger structures and plants.

Example 29

Regeneration of protoplasts of fertilized egg cells of Alexis by cocultivation with an embryogenic microspore culture: The effect of cocultivation versus cultivation in medium only.

Protoplasts of the fertilized egg were isolated from two spikes of **Alexis, 6 hours** after pollination as described in examples 1 and 18.

M1: Ten protoplasts were transferred in Kao 60 to a 12 mm Transwell and cocultivated with a 3 days old microspore culture transferred to a 12 well plate (cocultivation technique B).

M2: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cultured in Kao 90 + 1 mg/l 2,4-D with a 3 days old microspore culture in a 12 well plate (cocultivation technique B)..

M3: Five protoplasts were transferred in Kao 90 to a 12 mm Transwell and cultured in Kao 90 + 1 mg/l 2,4-D in a 12 well plate.

M4: Protoplasts were embedded in 1.5% LMP agarose and the next day cubes of agarose containing the protoplasts were cut out and cocultivated with a four day old microspore culture grown in Kao 90 + 1 mg/l 2,4-D (cocultivation technique B)..

	Culture method	No. of protoplasts	No of structures developing after 21 days	No of plants after 55 days
M 1	3 days old microspore culture	10	7	2
M 2	3 days old microspore culture + 1mg/l 2,4-D	10	10	10
M 3	Kao 90 + 1 mg/l 2,4-D	5	0	0
M 4	Agarose + 3 days old microspore culture + 1 mg/l 2,4-D	4	1	0

Conclusion: The observation on the regeneration of protoplast of the spring barley variety Alexis are identical to those for Igri. A better regeneration is obtained if non-embedded protoplasts are cultured directly and it is not possible to regenerate larger structures from protoplasts if these are not cocultivated. There appears to be a higher regeneration frequency if the protoplasts are cocultivated in Kao90 with 1 mg/l 2,4-D.

Example 30

Regeneration of protoplasts of fertilized egg cells of Alexis by cocultivation with an embryogenic microspore culture: The effect of using 2,4-D in the medium.

Protoplasts of the fertilized egg were isolated from two spikes of **Alexis, 20 hours** after pollination as described in examples 1 and 18.

M1: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a 4 days old microspore culture grown in Kao 60 and transferred to a 12 well plate (cocultivation technique B).

M2: Ten protoplasts were transferred in Kao 90 to a 12 mm Transwell and cultured in Kao 90 + 1 mg/l 2,4-D with a 4 days old microspore culture in a 12 well plate (cocultivation technique B).

	Culture method	No. of protoplasts	No. of developing structures after 21 days	No. of plants after 48 days
M 1	Kao 60	10	5	3
M 2	Kao 90 + 1 mg/l 2,4-D	10	7	7

Conclusion: There appears to be a better development of the protoplasts if they are cocultivated using 1 mg/l 2,4-D.

Example 31

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture, embryogenic suspension cultures and non-embryogenic suspension cultures.

Protoplasts of the fertilized egg were isolated from two spikes of **Igri, 5 hours** after pollination as described in examples 1 and 18.

M1: Six protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a two months old embryogenic microspore derived suspension culture in 12 well plate.

M2: Six protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a two months old non-embryogenic microspore derived suspension culture in 12 well plate.

M3: Five protoplasts were transferred in Kao 90 to a 12 mm Transwell and cocultivated with a three day old microspore preparation grown in a 12 well plate.

	Culture method	No. of protoplasts	No. of developing structures after 21 days	No. of plants after 48 days
M 1	Embryogenic suspension	6	5	1
M 2	Non-embryogenic suspension	6	1	0
M 3	3 days old microspore culture.	5	5	1

Conclusion: The best growth and regeneration of the egg protoplasts was observed by cocultivation with microspores. There was also a good growth and regeneration by cocultivation with the microspore derived embryogenic suspension while the non-embryogenic suspension was largely ineffective in supporting protoplast development.

Example 32

Fertility of Igri plants regenerated from protoplasts of the fertilized egg.

Plantlets derived from egg protoplasts of **Igri** were vernalized, transferred to soil and grown in a greenhouse as described in example 1. The regenerated plants were indistinguishable from control plants of the same variety grown from seeds. The first 15 spikes on the first 13 regenerants were analyzed to measure the degree of fertility in each spike.

	FERTILITY IN SPIKES %			
Plant number	100 %	75%	50%	25%
1	Spikes 1-15			
2	Spikes 1-15			
3	Spikes 1-10	Spikes 11-15		
4	Spikes 1-15			
5		Spike 1	Spikes 2-15	
6	Spikes 1-15			
7	Spikes 1-15			
8	Spikes 1-15			
9	Spikes 1-8	Spikes 9-15		
10	Spikes 1-12	Spikes 13-15		
11	Spikes 1-7	Spikes 8-15		
12			Spikes 1-12	Spikes 13-15
13	Spikes 1-15			

Example 33

Regeneration of protoplasts isolated from 36 day old microspore derived embryos by cocultivation with an embryogenic microspore culture.

Embryos from a 36 day old microspore culture (weight 1.1g) were digested with 1% cellulase Onozuka RS (Yakult Pharmaceutical Ind. C. Ltd., 1-1-19 Higashi, Shinbashi, Minato-ku, Tokyo, Japan) + 1% Pectinase (Serva) + 0.1% Pectolyase Y23 (Seishin) in 3 mM MES (2(N-morpholino)ethane sulfonic acid) + 0.65 M mannitol + 7 mM $CaCl_2$. After three hours the protoplasts were harvested by filtration through a 70 μm filter, which removes residual, non-digested structures followed by centrifugation at 100 x g for 5 min using a swing out rotor.

The protoplasts were thereafter resuspended in 10 ml CPW-13 medium (27.2 mg/l KH_2PO_4, 101 mg/l KNO_3, 246 mg/l $CaCl_2$, H_20, 0.16 mg/l KI, 0.025 mg/l $CuSO_4$ with 130 g/l mannitol), filtered through a 45μm filter and centrifuged at 150 x g for five min. 4.1 million protoplasts were isolated and transferred to K8p medium (Kao and Michayluk 1975). The protoplasts were transferred to 24 mm Transwells (200.000 protoplasts in one ml per Transwell) and cocultivated with a 36 days old microspore culture. The microspore culture was at this stage transferred from Kao 60 to K8p medium. Other protoplast preparations were not cocultivated but were transferred to either medium only or medium consisting of one part new medium to one part of medium conditioned by microspore cultures. After 29 days the protoplasts were subcultured, i.e., half the microspore derived structures were removed from the conditioning cultures and fresh medium in the form of LMS with 80g/l maltose was added.

In the four preparations, where the protoplasts from the microspore derived embryos were cocultivated with an embryogenic microspore preparation, protoplast derived callus structures were obtained in three of the four cocultivations (prep. 1: 11 structures; prep. 2: 6 structures; prep 3: 17 structures). In none of the other preparations where the culture only took place in medium under different conditions or in conditioned medium was any formation of protoplast derived larger structures observed.

Plants were not regenerated from any of the protoplast derived structures in this experiment.

Example 34

Regeneration of protoplasts isolated from 36 day old microspore derived embryos by cocultivation with an embryogenic microspore culture.

Embryos from a 36 day old microspore culture (weight 1.1g) were digested with 1% cellulase Onozuka RS (Yakult Pharmaceutical Ind. C. Ltd., 1-1-19 Higashi, Shinbashi, Minato-ku, Tokyo, Japan + 1% Pectinase (Serva) + 0.1% Pectolyase Y23 (Seishin) in 3 mM MES (2(N-morpholino)ethane sulfonic acid) + 0.65 M mannitol + 7 mM $CaCl_2$. After three hours the protoplasts were harvested by filtration through a 70μm filter, which removes residual, non digested structures followed by centrifugation at 100 x g for 5 min using a swing out rotor.

The protoplasts were thereafter resuspended in 10 ml CPW-13 medium (27.2 mg/l KH_2PO_4, 101 mg/l KNO_3, 246 mg/l $CaCl_2$, H_20 0.16 mg/l KI, 0.025 mg/l $CuSO_4$ with 130 g/l mannitol), filtered through a 45μm filter and centrifuged at 150 x g for five min. 2.2 million protoplasts were isolated and transferred to K8p medium (Kao and Michayluk 1975). The protoplasts were transferred to 24 mm Transwells (200.000 protoplasts in one ml per Transwell) and cocultivated with a 36 days old microspore culture. The microspore culture was at this stage transferred from Kao 60 to K8p medium. Other protoplast preparations were not cocultivated but were transferred to either medium only or medium consisting of one part new medium to one part of medium conditioned by microspore cultures. After 29 days the protoplasts were subcultured, i.e., half the microspore derived structures were removed from the conditioning cultures and fresh medium in the form of LMS with 80g/l maltose was added.

In the two preparations, where the protoplasts from the microspore derived embryos were cocultivated with an embryogenic microspore preparation, protoplast derived callus structures were obtained in one of these (prep. 1: 13 structures). In none of the other preparations, where the culture only took place in medium under different conditions or in conditioned medium, was any formation of protoplast derived larger structures observed.

Nine green and eight albino plants were regenerated from the 13 protoplast derived structures. Three of the nine green plants died after transfer to the greenhouse. Of the six surviving green plants one was completely fertile, two partially fertile, one completely fertile while the two remaining plants not yet had formed spikes.

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Example 35

Regeneration of protoplasts of fertilized egg cells of Igri by cultivation in microspore conditioned medium.

Protoplasts of the fertilized egg were isolated from three spikes of **Igri, 8** hours after pollination.

M1: Eight protoplasts were transferred in Kao 90 and cultured in 1 ml conditioned medium in a 12 well plate. The medium (Kao90) had been conditioned by cultivation of a 7 days old microspore preparation in a 24 mm Transwell. Every 7th day half the medium was removed from the microspore preparation and replaced by fresh medium while the conditioned medium was transferred to the 12 well plate to be used for cultivation of the egg protoplasts.

M2: Eight protoplasts were transferred in Kao 90 and cultured in 1 ml conditioned medium in a 12 well plate. The medium (Kao90) had been conditioned by cultivation of a 7 days old microspore preparation in a 24 mm Transwell. Every 3rd. day half the medium was removed from the microspore preparation and replaced by fresh medium while the conditioned medium was transferred to the 12 well plate to be used for cultivation of the egg protoplasts.

M3: Eight protoplasts were transferred in Kao 90 and cultured in 1 ml conditioned medium in a 12 well plate. The medium (Kao90 + 1 mg/l 2,4-D) had been conditioned by cultivation of a 10 days old microspore preparation in a 24 mm Transwell. Every 7th day half the medium was removed from the microspore preparation and replaced by fresh medium while the conditioned medium was transferred to the 12 well plate to be used for cultivation of the egg protoplasts.

M4: Six protoplasts were transferred in Kao 90 and cultured in 1 ml conditioned medium in a 12 well plate. The medium (Kao90 + 1 mg/l 2,4-D) had been conditioned by cultivation of a 10 days old microspore preparation in a 24 mm Transwell. Every 3rd. day half the medium was removed from the microspore preparation and replaced by fresh medium while the conditioned medium was

transferred to the 12 well plate to be used for cultivation of the egg protoplasts.

M5: Twelve protoplasts were transferred in Kao 90 and cultured in 1 ml conditioned medium in a 12 well plate. The medium (Kao90) had been conditioned by cultivation of a 22 days old microspore preparation in a 24 mm Transwell. Every day half the medium was removed from the microspore preparation and replaced by fresh medium while the conditioned medium was transferred to the 12 well plate to be used for cultivation of the egg protoplasts.

Observation: Growth and development of egg protoplasts was only observed when they were cultured in conditioned medium that had been replaced every day. There was thus no growth if the medium had been replaced by conditioned medium every 3rd or 7th day.

Example 36

Regeneration of protoplasts of fertilized egg cells of Igri by cocultivation with an embryogenic microspore culture: The effect of the length of the cocultivation period..

Protoplasts of the fertilized egg were isolated from three spikes of **Igri, 6** hours after pollination. The ovaries were dissected in Kao90 and the protoplasts isolated in Kao 90 + 1 mg/l 2,4-D. The protoplasts were transferred in Kao90 + 1 mg/l 2,4-D to four 12 mm Transwells and cocultivated with a 5 days old microspore culture for 9, 16, 23 og 30 days, whereafter the Transwells with the developing structures were transferred to Kao 90 + 1mg/l 2,4-D. When the embryogenic structures had reached about 2 mm in length they were transferred to Kao 90-agarose for further development and germination.

	Cocultivation days	No. of protoplasts	No. of developing structures after 21 days	No. of plants after 57 days	No. of plants after 73 days
M 1	9 days	5	4	0	0
M 2	16 days	5	5	0	0
M 3	23 days	6	5	0	6
M 4	30 days	6	5	7	7

Conclusion: Cocultivation for 9 days results in a very limited growth and development of the protoplasts while cocultivation for 16 days gives rise to small, loose callus structures. Cocultivation for 23 and 30 days resulted in a high frequency of plant regeneration, but the fastest development was observed when a 30 days period of cocultivation was used.

Example 37

Regeneration of protoplasts of fertilized egg cells of ***Igri*** *by cocultivation with an embryogenic microspore culture: The effect of agarose embedding.*

Protoplasts of the fertilized egg were isolated from two spikes of **Igri, 18** hours after pollination. The ovaries were dissected in Kao 90 and the protoplasts isolated in Kao90 + 1mg/l 2,4-D.

M1: 10 protoplasts were transferred in Kao 90 + 1 mg/l 2,4-D to 24 mm Transwells and cocultivated with a 7 days old microspore culture in 6 wells plates.

M2: 10 protoplasts were embedded individually in 0.75% LMP agarose in Kao90 on cover slips (2μl Kao90 containing the protoplast was mixed with 5μl 0.75% LMP agarose in Kao90) . The cover slides had previously been cleaned in 96% ethanol, dried and sterilized by uv irradiation for a minimum of 2 hours. After the agarose had solidified 0.5 ml Kao90 + 1 mg/l 2,4-D was added to the cover slip, the agarose beads were loosened from the glass surface and thereafter washed down into a 24 mm Transwell and cocultivated with a 7 days old microspore culture in 6 wells plates.

	Culture-method	No. of protoplasts	No. of developing structures after 21 days	No. of plants after 47 days	No. of plants after 82 days
M 1	Liquid medium	10	7	2	3
M 2	Embedded in agarose	10	14	6	16

Conclusion: The experiment shows that by embedding in a thin agarose solution (0.75%) it is possible to obtain better regeneration than when using liquid medium. The better results obtained in this experiment compared to the results described in experiment 2 may be attributed to the low agarose concentration in the present example, which may facilitate a better penetration of compounds secreted from the microspores through the agarose into the egg protoplasts.

Example 38

Analysis of wall formation in dividing egg protoplasts by staining with Calcofluor White.

Protoplasts of the fertilized egg were isolated from four spikes of **Igri** (two spikes **8** hours after pollination and two spikes **18** hours after pollination) The ovaries were dissected in Kao90 and the protoplasts isolated in Kao 90 and embedded in 0.75% LMP in Kao90 as described in example 37.

M1: Ten protoplasts were isolated from one spike 8 hours after pollination and ten protoplasts from another spike 18 hours after pollination. 0.01% Calcofluor White in water was added to the cover slips. A control of the staining of the wall components, pollen grains as well as parts of the stigmata had been embedded as well. The preparations were analyzed under uv illumination in a light microscope. In none of the protoplasts were observed fluorescent material around the protoplasts, while there was clearly fluorescent wall material in the pollen grains and the stigmata.

M2: Ten protoplasts were isolated from one spike 8 hours after pollination and ten protoplasts from another spike 18 hours after pollination The protoplasts were culture in the agarose beads on the cover slip for five days whereafter 0.01% Calcofluor White in water was added to the cover slips. A control of the staining of the wall components pollen grains as well as parts of the stigmata had been embedded as well. The preparations were analyzed under uv illumination in a light microscope. All protoplasts had divided one or more times and there was a distinct fluorescent wall around the individual cells.

Example 39

Cocultivation of Alexis microspores with microspores of Igri.

In anther and microspore culture of the spring barley variety Alexis the number of regenerated plants is low with a high frequency of albino plants. In addition to the formation of a few embryos, there is extensive callus formation, but plants cannot be regenerated from these structures. To elucidate if Igri microspore cultures are able to support the development of Alexis microspores, microspores from this variety were cocultivated with Igri microspores. Microspores from Alexis were isolated as described for Igri in example 21 and transferred to 12 mm Transwells (5.2×10^4 microspores per Transwell) and cocultivated with 6.5×10^4 - 10×10^4 Igri microspores in 12 well plates. The number of developing structures were counted after six weeks.

The results described below are the mean of three separate experiments.

Culture method	No. of structures per Transwell
cocultivation	149
no cocultivation	79

Conclusion: Cocultivation of Alexis microspores with freshly isolated Igri microspores results in a doubling of the number of developing Alexis structures.

Example 40

Survival and development of microinjected egg protoplasts.

Protoplasts of the fertilized egg were isolated in Kao 90 from spikes of Igri (A135,138 and 139) and Alexis (A134. 136, 137, 139-167) at different stages after pollination and embedded in agarose on cover slips as described in example 37. Three different agarose dilutions were tried, namely 1.5% LMP agarose in Kao90 diluted with Kao90 1:1, water 1:1 and Kao 120 1:1. The embedded protoplasts were microinjected (se examples 15, 16 and 20) with linearized plasmid in 10 mM Tris + 0.1 mM EDTA, pH 7.8 (Linearized plasmids: pEMU-GUS-NOS, example 15) and (pACT-GUS-NOS (ACT: actin promoter, see Zhang et al.1991)) linearized by cutting with ScaI in the plasmid sequence. The microinjected protoplasts were analyzed 60 min after the injection for survival and the agarose droplets were thereafter washed down in 24 mm Transwells and cocultivated with microspores as described in example 37. For cocultivation were used 1-10 days old microspore preparations.

The results are given in the table on the following page. nd, not yet determined

Conclusion: On average 69% of the protoplasts survive the injection and of these 52% continue development. In the few experiments where plantlets until now have been regenerated, 59 plants have been regenerated from 58 embryogenic structures.

Experiment no.	Time after pollination hours	Dilution medium	Plasmid	DNA concentration	No.of protoplasts injected	No. of surviving protoplasts	No.of developing structures	No. of plantlets
A134	7	Kao90	EMU	100ng/µl	12	6	2	nd
A135	22	Kao90	EMU	50ng/µl	11	5	1	nd
A136	22	Kao90	EMU	50ng/µl	11	5	5	nd
A137	22	Kao90	EMU	25ng/µl	12	8	7	nd
A138	18	Kao90	EMU	6.25ng/µl	14	4	2	nd
A139	5	Kao90	EMU	6.25ng/µl	13	3	2	nd
A140	22	Kao90	ACT	50ng/µl	9	4	0	nd
A141	8	Kao90	ACT	25ng/µl	21	12	5	7
A142	19	water	ACT	12.5ng/µl	13	8	6	1
A143	8	water	ACT	6.25ng/µl	15	12	8	4

A144	8	water	ACT	50ng/µl	18	9	4	9
A145	8	water	ACT	6.25ng/µl	15	9	8	4
A147	8	Kao120	ACT	6.25ng/µl	15	12	5	3
A148	21	Kao120	ACT	10ng/µl	7	5	3	nd
A149	6	Kao120	ACT	10ng/µl	21	17	9	nd
A150	6	water	ACT	10ng/µl	15	9	8	13
A151	19	Kao120	ACT	10ng/µl	9	9	3	2
A152	6	Kao120	ACT	10ng/µl	26	18	11	16
A 153	6	Kao120	ACT	10ng/µl	17	11	5	nd
A154	19	Kao120	ACT	10ng/µl	4	3	2	nd
A155	6	Kao120	ACT	10ng/µl	19	12	7	nd
A156	19	Kao120	ACT	10ng/µl	9	8	4	nd
A157	6	Kao120	ACT	10ng/µl	20	17	6	nd
A158	6	Kao120	EMU	5ng/µl	26	21	9	nd

A159	19	Kao120	EMU	5ng/µl	11	7	4	nd
A160	6	Kao120	EMU	5ng/µl	16	14	10	nd
A161	19	Kao120	ACT	1.5ng/µl	13	19	5	nd
A162	5	Kao120	ACT	1.5ng/µl	23	19	7	nd
A164	18	Kao120	ACT	1.5ng/µl	13	9	5	nd
A165	5	Kao120	ACT	1.5ng/µl	17	12	1	nd
A166	6	Kao120	ACT	1.5ng/µl	22	15	10	nd
A167	6	Kao90	ACT	1.5ng/µl	23	18	14	nd

Example 41

Transformation of egg protoplasts by microinjection

Protoplasts of the fertilized egg were isolated in Kao 90 from two spikes of Alexis 6 hours after pollination and embedded in agarose on cover slips as described in example 37. The embedded protoplasts were thereafter microinjected (see examples 15, 16 and 20) with 25 ng/µl pEMU-GUS-NOS plasmid in 10 mM Tris + 0.1 mM EDTA, pH 7.8. The microinjected protoplasts were analyzed for survival after 60 min and the agarose droplets with the protoplasts were thereafter washed down into 24 mm Transwells and cocultivated with a 17 days old microspore preparation. After 9 days the developing structures were analyzed by the histochemical assay for GUS expression as described in example 15.

No. of protoplasts injected	No. of developing structures after 9 days	No of structures with GUS expression
0	8	2

Conclusion. It is possible to introduce a reporter gene into the protoplasts and obtain expression.

Example 42

Regeneration of wheat plants from fertilized eggs cultured in intact ovules

Pollinated spikes of the wheat variety Walter were sterilized by spraying with 96 % ethanol, whereafter they were allowed to dry. The ovaries with the palea were dissected out and placed in a sterile dish and transferred one by one to Kao 90 for dissection. The ovaries were placed with their dorsal side up (containing the vascular strand) and cleaved by inserting the fine pointed legs of a forceps. The ovules were dissected out and transferred to Kao90-agarose.

	Time after pollination	No. of ovules	No. of ovules with embryo formation	No. of plants after 46 days
M1	26	13	1	1
M2	8	18	1	1
M3	8	14	1	1

The regenerated plants were transferred to the greenhouse and grown to maturity. All the regenerants were normal and formed about 10 spikes each, all 100% fertile.

Example 43

Cultivation and regeneration of protoplasts of fertilized eggs of wheat.

M1 Ovaries were isolated from an unpollinated spike of the wheat variety Walter and sterilized as described for barley in example 1. The ovaries were dissected in Kao 90 as described for barley in example 1 and the protoplasts isolated and embedded in 0.75% LMP agarose-Kao90, 1:1 on a sterile cover slip as described in example 37. The cover slips were placed in a Falcon Primaria Organ Tissue Culture Dish #3037 and sealed with Nescofilm. Water was added to the outer chamber to prevent desiccation of the agarose. The protoplasts were photographed and analyzed daily. None of the protoplasts divided and after five days all had degenerated.

M2 Two protoplasts of fertilized egg cells were isolated from ovaries from a pollinated spike of Walter, 16 hours after pollination. The sterilization, isolation and cultivation was performed as described above. Both protoplasts divided several times over the following seven days and formed microcalli consisting of a few cells, whereafter they degenerated.

M3 Ten protoplasts were isolated from ovaries from a pollinated spike of Walter, 8 hours after pollination as described above and transferred to Kao90 in a 12 mm Transwell and cocultivated with a 13 days old microspore preparation. Three of the protoplasts developed into 1-2 mm large structures.

M4 Five protoplasts were isolated from ovaries of a pollinated spike of Walter, 26 hours after pollination as described above and transferred to Kao90 in a 12 mm Transwell and cocultivated with a 13 days old microspore preparation. One of the protoplasts developed into an embryo that germinated into a normal plantlet. The plantlet was grown to maturity and developed into a completely normal plants forming about 10 spikes which were 100 % fertile

Conclusion: It is possible to isolate protoplasts of unfertilized and fertilized eggs of wheat. As in barley unfertilized eggs are unable to divide while protoplasts of the fertilized egg can divide several times if grown in vitro. By cocultivation with embryogenic microspores of barley, protoplasts of fertilized egg can develop into larger structures such as embryos and into completely normal plants.

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CLAIMS:

1) A method of regeneration of plant materials, including protoplasts, cells and tissues to plants, **characterized** in

that the protoplasts, the cells or the tissues are cocultivated with microspores or anthers or are cultivated in a medium containing extracts of microspores or anthers or a medium in which there has been cultured microspores or anthers or a medium containing fractions of a medium in which there has been cultured microspores or anthers or

that the plant materials in the cases where they consist of microspores or anthers are cocultivated with microspores or anthers from another variety or species or are cultured in a medium containing extracts of microspores or anthers from another variety or species or a medium in which there has been cultured microspores or anthers of another variety or species or a medium containing fractions of a medium in which there has been cultured microspores or anthers from another variety or species.

2) Method according to claim 1, **characterized** by the following steps

a) selection of microspores or anthers which are disposed for regeneration,

b) cultivation of the selected microspores or anthers in a culture medium,

c) cultivation of the protoplasts, the cells and the tissues to be regenerated in the medium mentioned in step b or in a medium containing fractions of the medium mentioned in step b.

3) Method according to claims 1 and 2, **characterized** by the following steps

a) selection of microspores or anthers which are disposed for regeneration,

b) cultivation of the selected microspores or anthers in a culture medium for embryogenic growth

c) removal of the cultured microspores or anthers from the culture medium preferably by filtration,

d) cultivation of the protoplasts, the cells or the tissues to be regenerated until germination in the medium mentioned in step c or a medium containing fractions of the medium mentioned in step c.

4) Method according to claim 1, **characterized** by the following steps,

a) selection of microspores or anthers which are disposed for regeneration,

b) cocultivation of the protoplasts, the cells and the tissues to be regenerated until germination with the selected microspores or anthers in step a.

5) Method according to claim 1, **characterized** by the following steps

a) selection of microspores or anthers which are disposed for regeneration

b) maceration of the selected microspores or anthers in step a, preferably in a liquid medium and preferably by blending and possibly followed by filtration or fractionation or addition of essential nutritional compounds to make a culture medium if these are not already present.

c) cultivation of the protoplasts, the cells or the tissue to be regenerated until germination in the culture medium described in step b or in a culture medium containing the filtrate described in step b or the medium containing microspores or anthers described in step b.

6) Method according to claims 1-5, **characterized** in that the protoplasts, the cells or the tissue to be regenerated are cultured in a liquid, semi-liquid or solid medium which can be penetrated by or is in contact with a medium in which microspores or anthers are cultured or a medium in which microspores or anthers have been cultivated for embryogenic growth or a medium containing extracts of microspores or anthers.

7) Method according to claims 1-6, **characterized** in that the microspores or the anthers are from the barley variety Igri.

8) Method according to claims 1-7, **characterized** in that the protoplasts are egg protoplasts or protoplasts from embryogenic cells.

9) Method according to claims 1-7, **characterized** in that the cells are single cells or cell aggregates, preferably embryogenic cells.

10) Method according to claims 1-7, **characterized** in that the tissues are whole organs, preferably zygotic embryos or microspores.

11) Method according to claims 1-10, **characterized** in that the protoplasts, the cells or the tissue to be regenerated are from angiosperms, preferably monocotyledonous plants and in particular plants from the grass family (Gramineae).

12) Method according to claim 10, **characterized** in that the protoplasts, the cells or the tissues to be regenerated are from plants belonging to the group constituted by barley, wheat, Sorghum, maize and rice, preferably barley and wheat.

13) Method of producing transgenic plants, **characterized** by the following steps

a) isolation of protoplasts, cells and tissues

b) introduction of foreign genetic materials in the plants materials described in step a, or in the cases the protoplasts are from unfertilized eggs possibly by in vitro fertilization

with sperm nuclei from another species or variety, preferably by microinjection, electroporation, polyethylene glycol or laser and in particular by microinjection

c) regeneration of the transformed cells or the in vitro fertilized protoplasts, cells or tissues using the methods according to claims 1-12.

14) Method of producing hybrids or cybrids, **characterized** by the following steps

a) isolation of the first protoplast

b) isolation of a second protoplast or a sperm cell and if wanted the removal of the nucleus from the first or the second protoplast,

c) fusion of the two protoplasts preferably by polyethylene glycol treatment or by electro-fusion

d) regeneration of the fused cells to hybrids or cybrids by using the method in claims 1-12

15) Method of producing of homozygous plants, **characterized** by the following steps

a) isolation of haploid protoplasts or cells which optionally can be induced to undergo chromosome doubling preferably by treatment with colchicine,

b) regeneration of the double haploid protoplasts or cells to homozygous plants by the procedure in claims 1-12.

16) Method of producing of plants with special properties, **characterized** by the following steps

a) isolation of protoplasts or single cells and cultivation of these

b) in vitro selection of the protoplasts or the single cells or cultures of these

c) regeneration of the protoplasts or single cells selected in step b to whole plants with special properties by using the method according to claims 1-12.

17) Transgenic plants, hybrids or cybrids, homozygous plants or plants with special properties produced by the methods according to claims 13, 14, 15 or 16.

18) Regenerable parts of plants or progeny from transgenic plants, hybrids or cybrids, homozygous plants or plants with special properties according to claim 17.

19) Medium to be used for regeneration of protoplasts, cells or tissue and containing essential nutrients for the material to be regenerated, **characterized** by that the medium also contains extracts of microspores or anthers or components secreted by the microspores or the anthers during their culture, preferably an embryogenic culture.

20) Method of isolating egg protoplasts from ovules, **characterized** by the following steps:

a) removal of the integuments from at least the micropylar part of an ovule,

b) puncturing the vacuole of the central cell

c) a careful removal of the nucellus tissue at least from the micropylar part of the ovule

d) isolation of the egg protoplast and removal of protoplasts of the synergids

and where at least steps c-d, and particularly steps b-d takes place in a medium which is not harmful to the protoplast.

21) Method of isolating synergid protoplasts, **characterized** by the following steps

a) removal of the integuments from at least the micropylar part of the ovule,

b) puncturing the vacuole of the central cell

c) a careful removal of the nucellus tissue at least from the micropylar part of the ovule,

d) isolation of the synergid protoplast and removal of the egg protoplasts

and where at least steps c-d, and particularly steps b-d takes place in a medium which is not harmful to the protoplast.

22) Method according to claims 20 or 21, **characterized** in that the medium has an osmotic pressure between 250-500 mOsm and in particular a pressure of 375 mOsm.

23) Method according to claims 20-22, **characterized** in that the ovule is fertilized

24) Method according to claims 20-22, **characterized** in that the ovule is unfertilized

25) Method according to claims 20-24, **characterized** by that the ovule is from an angiosperm, preferably from a monocotyledonous species and especially from the grass family (Gramineae).

26) Method according to claims 20-25, **characterized** in that the ovule is from barley, wheat, Sorghum, maize or rice, preferably barley or wheat.

27) Egg protoplasts and synergid protoplasts isolated by the method according to claims 20-26 and cultures of these.

28) Method of producing transgenic plants, **characterized** by the following steps

a) isolation of egg protoplasts by using the methods according to claims 20 or 22-26,

b) introduction of foreign genetic materials in the plants materials described in step a, or in the cases the protoplasts are from unfertilized eggs possibly by in vitro fertilization with sperm nuclei from another species or variety, preferably by microinjection, electroporation, polyethylene glycol or laser and in particular by microinjection.

c) regeneration of the transgenic egg protoplasts preferably by using the method according to claims 1-12.

29) Method of producing hybrids or cybrids, **characterized** by the following steps:

a) isolation of the first protoplast by using the methods according to claims 20 or 22-26

b) isolation of a second protoplast or a sperm cell and if wanted the removal of the nucleus from the first or the second protoplast,

c) fusion of the two protoplasts preferably by polyethylene glycol treatment or by electro-fusion,

d) regeneration of the fused cells to hybrids or cybrids by using the method in claims 1-12

30) Method of producing of homozygous plants, **characterized** by the following steps

a) isolation of unfertilized egg protoplasts and possibly an induction of chromosome doubling preferably by treatment with colchicine,

b) regeneration of the double haploid protoplasts or cells to homozygous plants by the procedure in claims 1-12.

31) Method of producing plants with special properties, **characterized** by the following steps

a) isolation of egg according to claims 20 or 22-26 and the culture of these

b) in vitro selection of the egg protoplasts or cultures of these

c) regeneration of the protoplasts selected in step b to whole plants with special properties by using the method according to claims 1-12.

32) Transgenic plants, hybrids or cybrids, homozygous plants or plants with special properties produced by the procedures according to claims 28, 29, 30 or 31.

33) Regenerable parts of plants from or progeny of transgenic plants, hybrids or cybrids, homozygous plants or plants with special properties according to claims 31.

34) Method of in situ transformation of fertilized eggs or embryos, smaller than 200μm i diameter, **characterized** by the following steps:

a) isolation of an ovary and exposure of at least the micropylar part of the ovule by dissecting away the surrounding ovary tissue and removal of the integuments around at least the micropylar part of the ovule

b) the introduction of foreign genetic material by microinjection

35) Method according to claim 34, **characterized** in that the ovary is from an angiosperm, preferably from a monocotyledonous plant and especially from a plant belonging to the grass family (Gramineae).

36) Method according to claim 35, **characterized** in that the ovary is from either barley, wheat, Sorghum, maize or rice, preferably from barley or wheat.

37) Method of producing transgenic plants, **characterized** by the following steps:

a) transformation in situ of egg cells or embryos by using the method according to claims 34-36,

b) regeneration of the transformed egg cells or embryos by cultivation of whole ovules or the tips of ovules containing the egg cells or the embryos

38) Transgenic plants produced by the method according to claim 37.

39) Regenerable parts of plants from or progeny of transgenic plants according to claim 38.

COCULTIVATION OF ZYGOTE PROTOPLASTS WITH AN EMBRYOGENIC MICROSPORE PREPARATION USING TRANSWELL MEMBRANE RAFTS

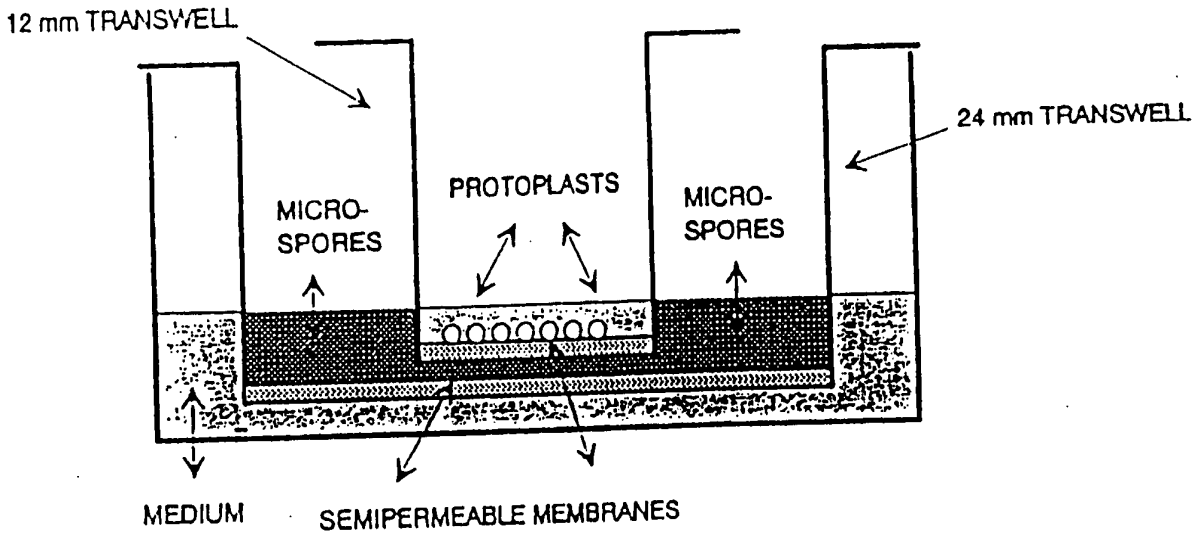

Figure 1. Cocultivation cell according to method A

PCT/DK93/00246

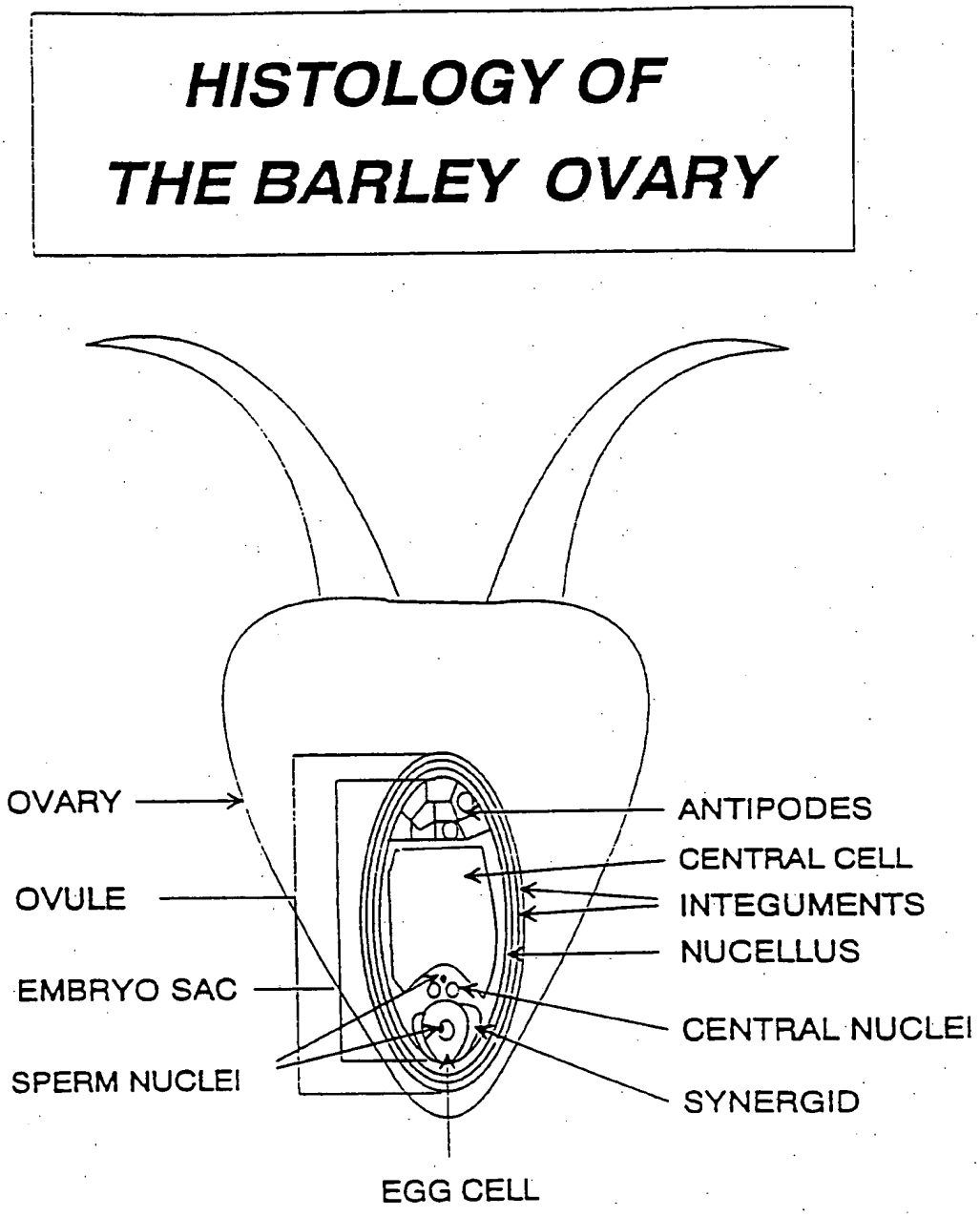

Figure 2. Diagram of the barley ovary, the ovule and the embryo sac

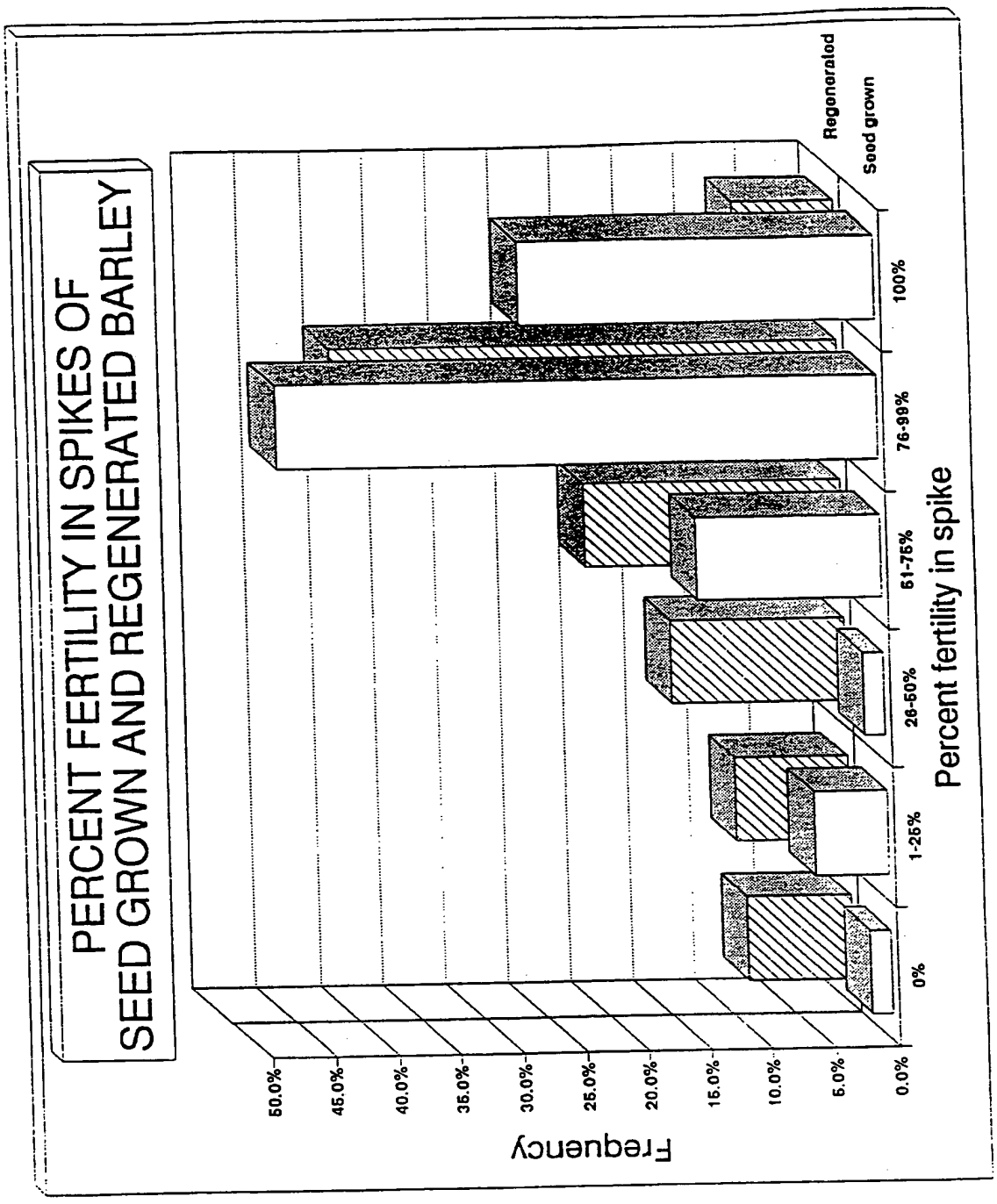

Figure 3. Percent fertility in spikes

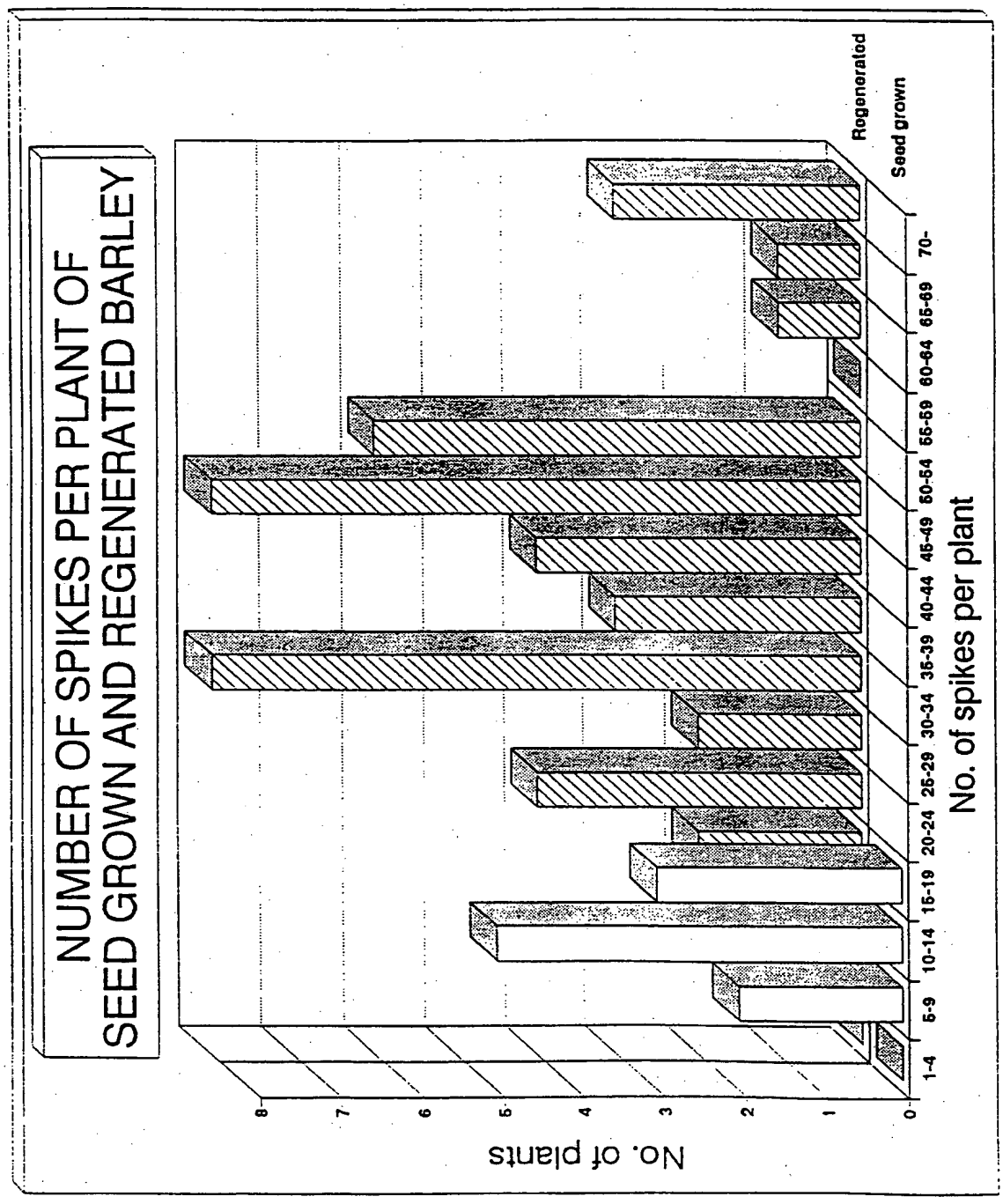

Figure 4. Number of spikes per plant

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00246

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A01H 4/00, C12N 5/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A01H, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 357,Biotechnology Abstracts, Dialog acccession no.102406, DBA Accession no. 90-05097, Lazar M D et al: "The effects of culture environment with genotype on wheat (Tritioum aestivum) anther culture response - culture medium and temperature pretreatment effect on callus culture induction and embryogenesis", Plant Cell Rep., 8,9, 525-29 --	1,5-19,28-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1993	17 -12- 1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer MIKAEL G:SON BERGSTRAND Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00246

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 5, Dialog accession no. 5301379, BIOSIS Number: 81068686, Koehler F et al: "Regeneration of isolated barley microspores in conditioned media andrials to characterize the responsible factor", J Plant Physiol 121 (2), 1985 (Recd. 1986) 181-192	17-18,32-33, 38-39
A		1-16,19, 28-31,34-37
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X	J.PLANT PHYSIOL., Volume 139, 1991, Sergio J. Ochatt, "Strategies for Plant Regeneration from Mesophyll Protoplasts of the Recalcitrant Fruit and Farmawoodland Species" page 155 - page 160	17-18,32-33, 38-39
A		1-16,19, 28-31,34-37
	--	
A	Dialog Information Services, file 5, BIOSIS, Dialog accession no. 10101443, Biosis number: 95101443, Faure J-E et al: "Ultrastructural characterization and three-dimensional reconstruction of isolated maize zea-mays L. egg cell protoplasts", Protoplasma 171 (3-4), 1992, 97-103	20-39
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A	Dialog Information Services, file 5, BIOSIS, Dialog accession no. 5441956, Biosis number: 82086759, Mol R: "Isolation of protoplasts from female gametophytes of torenia-fournieri", Plant Cell Rep 5 (3), 1986, 202-206	20-39
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A	Dialog Information Services, file 5, Biosis, Dialog accession no. S252420, Biosis number: 81019727, Hu S-Y et al: "Isolation of viable embryo sacs and their protoplasts of nicotiana-tabacum", Acta Bot Sin 27 (4), 1985, 337-344	20-39
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00246

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	R A Dixon, "Plant cell culture, a practical approach", 1985, IRL Press, (Oxford), page 38 - page 44; page 95 - page 96	20-39

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK93/00246

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See attached sheet!

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

I Claims 1-19 completely, claims 28-39 partially:
Methods and media for regenerating plant materials

II Claims 20-27 completely, claims 28-39 partially
Methods for isolating protoplasts

The special technical feature of group I relates to coculturing protoplasts, plant cells or plant tissues with microspores or anthers or extracts thereof in order to regenerate plants. The special technical feature of group II is related to the removal of the integuments and nucellus tissue from at least the micropylar part of an ovule. These groups of inventions are not so linked as to form a single general inventive concept.